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(54) Title: RECOMBIANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS, CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF

(57) Abstract

An adenovirus E1/E4 expressing packaging cell line is provided, which permits the generation of recombinant adenoviruses deleted in both gene regions. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell is provided by infecting a target cell with a recombinant AAV comprising a selected transgene under the control of regulatory sequences. The infected cell is contacted with an agent which facilitates the conversion of single stranded recombinant virus to its double stranded form.

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RECOMBINANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS,
CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF

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Field of the Invention

The present invention relates generally to the field of somatic gene therapy, and specifically to methods and 10 compositions useful in the treatment of genetic disorders.

Background of the Invention

Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a therapeutic or reporter 15 transgene to a variety of cell types [see, e.g., M. S. Horwitz et al, "Adenoviridae and Their Replication", Virology, second edition, pp. 1712, ed. B. N. Fields et al, Raven Press Ltd., New York (1990)]. Recombinant adenoviruses (rAds) are capable of providing extremely 20 high levels of transgene delivery to virtually all cell types, regardless of the mitotic state. The efficacy of this system in delivering a therapeutic transgene *in vivo* that complements a genetic imbalance has been demonstrated in animal models of various disorders [K. F. 25 Kozarsky et al, Somatic Cell Mol. Genet., 19:449-458 (1993) ("Kozarsky et al I"); K. F. Kozarsky et al, J. Biol. Chem., 269:13695-13702 (1994) ("Kozarsky et al II) and others]. The use of recombinant adenoviruses in the transduction of genes into hepatocytes *in vivo* has 30 previously been demonstrated in rodents and rabbits [see, e.g., Kozarsky II, cited above, and S. Ishibashi et al, J. Clin. Invest., 92:883-893 (1993)].

The first-generation recombinant, replication-deficient adenoviruses which have been developed for gene 35 therapy contain deletions of the entire E1a and part of the E1b regions. This replication-defective virus is

grown on an adenovirus-transformed, complementation human embryonic kidney cell line containing a functional adenovirus Ela gene which provides a transacting Ela protein, the 293 cell [ATCC CRL1573]. El-deleted viruses 5 are capable of replicating and producing infectious virus in the 293 cells, which provide Ela and Elb region gene products in trans. The resulting virus is capable of infecting many cell types and can express the introduced gene (providing it carries its own promoter), but cannot 10 replicate in a cell that does not carry the El region DNA unless the cell is infected at a very high multiplicity of infection.

Adeno-associated virus (AAV) is an integrating human DNA parvovirus which has been proposed for use as a gene 15 delivery vehicle for somatic gene therapy [B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168 (1990)]. This small non-enveloped virus contains a 4.6 kb single stranded (ss) DNA genome that encodes sets of regulatory and capsid genes called 20 rep and cap. Rep polypeptides (rep78, rep68, rep62 and rep40) are involved in replication, rescue and integration of the AAV genome. The cap proteins (VP1, VP2 and VP3) form the virion capsid. Flanking the rep and cap open reading frames at the 5' and 3' ends are 145 25 bp inverted terminal repeats (ITRs), the first 125 bp of which are capable of forming Y- or T-shaped duplex structures.

Recombinant forms of AAV (rAAV) have been developed as vectors by replacing all viral open reading frames 30 with a therapeutic minigene, while retaining the necessary cis elements contained in the ITRs. [See, e.g., US Patent Nos. 4,797,368; 5,153,414; 5,139,941; 5,252,479; and 5,354,678; and International Publication Nos. WO 91/18088 published November 28, 1991; WO 93/24641 35 published December 9, 1993 and WO94/13788 published June

23, 1994]. However, progress towards establishing AAV as a transducing vehicle for gene therapy has been slow for a variety of reasons. For example, the integrated provirus preferentially targets specific sites in 5 chromosome 19. Additionally, difficulties surround large-scale production of replication defective recombinants. The cells employed to produce rAAV must also be infected with adenovirus or herpesvirus to provide the necessary helper functions, thereby producing problems in purifying 10 recombinant AAV (rAAV) from contaminating virus in culture. Practical experience with purified recombinant AAV as a gene therapy vector has been disappointing, because the more purified the AAV is from co-infection 15 with its helper virus in culture, the lower the gene transduction efficiencies that the rAAV displays.

There remains a need in the art for additional recombinant adenoviruses and rAAV, therapeutic compositions and methods which enable effective use of these recombinant viruses in the treatment of disorders 20 and diseases by gene therapy.

Summary of the Invention

In one aspect of this invention, a packaging cell line is provided which expresses adenovirus genes E1a, E1b and E4, or functional fragments thereof, e.g., the E4 25 open reading frame (ORF) 6.

In another aspect, the invention provides a rAd comprising the DNA of at least a portion of the genome of an adenovirus having functional deletions of the E1 and E4 gene regions; a suitable gene operatively linked to 30 regulatory sequences directing its expression, and an adenovirus capsid, the rAd capable of infecting a mammalian cell and expressing the gene product in the cell *in vivo* or *in vitro*. The invention also provides a mammalian cell infected with the rAd described above.

In still another aspect, the invention provides a rAd shuttle vector comprising the DNA of at least a portion of the genome of an adenovirus having functional deletions of the E1 and E4 gene regions.

5 In a further aspect, the invention provides a method for producing the above-described recombinant Ad and a method for delivering a selected gene into a mammalian cell using the recombinant Ad described above.

10 In another aspect, the invention provides a method for enhancing the efficiency of transduction of a recombinant AAV into a target cell. The method operates, in brief, by infecting a target cell with a ss recombinant adeno-associated virus (rAAV) which comprises a transgene operatively linked to regulatory sequences 15 directing its expression, and contacting the infected cells with an agent which facilitates the conversion of ss rAAV to its double stranded (ds) form. Conversion of ss rAAV to ds rAAV occurs in the target cell, resulting in enhanced transduction of the rAAV into the target 20 cell. The agent may be a helper virus which carries a selected gene or functional fragment thereof encoding a polypeptide capable of enhancing the conversion of the ss rAAV to ds rAAV and which is co-infected into the same target cell. The agent may also be a drug or chemical 25 composition which accomplishes the same function and is applied to the infected target cell. This method can operate both in an ex vivo setting and in vivo.

30 In yet another aspect, the invention provides a novel recombinant AAV, which contains both the transgene intended for use in treating a genetic disease or disorder and at least one additional gene operatively linked to inducible or constitutive regulatory sequences. The additional gene(s) encodes a polypeptide capable of facilitating, alone or in concert with other additional 35 genes, the conversion of ss rAAV to its ds form upon

expression. In a preferred embodiment, the additional gene is adenovirus E4 or a functional fragment thereof. Also disclosed is a method for enhancing the efficiency of transduction of the novel rAAV into a target cell.

5 The novel rAAV and methods of this invention are also useful in pharmaceutical compositions for use in *ex vivo* and *in vivo* gene therapy treatment protocols for treating inherited diseases, cancer, and other genetic dysfunctions.

10 Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

15 Fig. 1 is a schematic drawing of an exemplary plasmid pMMTVE4ORF6 [SEQ ID NO: 1] or pMTE4ORF6, which contains an MMTV or sheep MT promoter, respectively, in control of a human E4 ORF 6 gene sequence, a growth hormone gene terminator sequence (GH), an SV40 ori, pBR322-based plasmid sequences including a *neo*^R gene, an 20 SV40 polyA site and an *amp*^R gene.

Fig. 2 is a schematic map of rAd H5.001CBLacZ [SEQ ID NO: 3] with indicated restriction endonuclease enzyme sites. The striated bar represents the CBLacZ minigene; the black bar represents Ad5 viral backbone, the 25 crosshatched bar represents Ad E4 deletion.

Fig. 3 plots *LacZ* forming units (LFU)/ml vs time (hours) for E4 complementing cell lines infected with H5.001CBLacZ.

30 Fig. 4A is a graph of the induction, ORF6 expression and viral production in 293-27-18 packaging cells plotting yield at 24 hours post-infection (pi) in LFU/ml and ORF6 protein (abs.mm) vs. concentration of the inducer, dexamethasone (μ M). Abs.mm is the intensity of the size of the protein band on a Western blot and 35 reflects absorbance and protein size in mm^2 . The square

is yield at 24 hours pi. The diamond is ORF6 protein detected at 24 hours pi.

Fig. 4B is a similar graph to that of Fig. 4A, except that the packaging cells are 293-10-3 cells. The 5 symbols are as described for Fig. 4A.

Fig. 5A is a bar graph plotting β -galactosidase enzyme activity in lysates from infected HeLa cells. The horizontal axis indicates the adenoviruses infected into the HeLa cells, with the symbol "+" indicating the 10 addition of the adenovirus to the rAAV, AV.CMVLacZ. The vertical axis indicates intracellular β -galactosidase specific activity (mUnits/mg protein) using ONPG. Below each bar, the fold-induction in specific activity 15 relative to cells that received the AV.CMVLacZ vector alone is given.

Fig. 5B is a bar graph plotting Ad multiplicity of infection (MOI) in HeLa cells of wild-type Ad5 or the E2 mutant dl802, the cells co-infected with rAAV vs. 20 intracellular β -galactosidase specific activity. See Example 11.

Fig. 6A is a graph in which β -galactosidase specific activity and counts per minute (CPM) are plotted along the vertical axis and adenovirus MOI's are on the horizontal axis for HeLa cells infected with wtAd5 and 25 rAAV according to Example 12. Data obtained from low MOI (1, 5, and 10) infections are shown.

Fig. 6B is a graph similar to that of Fig. 6A except that the cells were infected with Ad mutant dl802.

Fig. 7A illustrates a model for leading strand 30 synthesis of a complementary AAV strand in the presence of Rep (+Rep) or absence of Rep (-Rep). Rep expresses a terminal resolution activity that can convert a duplex structure with closed-ends to an open-ended duplex. In the absence of R p, terminal resolution is impaired 35 leaving the covalently closed, hairpin structures intact.

Under these conditions, hairpins are expected to be found leftward and rightward, since both strands of a rescued ds AAV genome are packaged into virions.

Fig. 7B is a schematic of linear AV.CMVLacZ with 5 labeled domains including the AAV ITRs, CMV immediate early enhancer/promoter (CMV), SV40 splice donor-splice acceptor (SD/SA), *E. coli* β -galactosidase cDNA (*LacZ*), and SV40 polyA signal (pA). Two NotI sites located at bp positions 1035 and 4509 are indicated.

Fig. 7C illustrates a closed end and an open end 10 fragment of rAV.CMVLacZ.

Figs. 7D, 7E and 7F indicate the mixture of open-ended and covalently closed duplex fragments generated by NotI digestion of ss AV.CMVLacZ at position 15 4509 in the absence of terminal resolution. The NotI 4509 digestion provides a convenient means of releasing a 361 bp fragment that contains the right ITR in the context of a hybridization target (i.e. SV40 pA). In the presence of terminal resolution, only the open-ended 361 20 bp fragment would be expected to be generated (Fig. 7D) by such digestion.

Fig. 8A is a bar graph plotting β -galactosidase specific activity (mUnits/mg protein) vs. increasing concentration of zinc (μ M) inducer for cell line 293 (MT-25 ORF6) transduced with AVCMVLacZ (first row below each bar). Also provided is the fold-induction relative to 293 cells (second row below each bar), and the fold-induction relative to 293(ORF6) cells maintained in the absence of zinc (third row).

Fig. 8B is a bar graph plotting CPM of duplex 30 monomer replicative form (RFm) of rAAV vs. the concentration of zinc (μ M) used for induction and the fold-induction relative to 293(ORF6) cells maintained in 0 mM zinc below each bar.

Fig. 8C is a graphical comparison of the induction profiles that describe AV.CMVLacZ transduction efficiency. Specific activity data from Fig. 8A and CPM data of AV.CMVLacZ RFm from Fig. 8B are plotted along the vertical axis, and concentration of zinc sulfate used during the experiment is shown along the horizontal axis.

Fig. 9 is a bar graph plotting specific activity (milliunits β -galactosidase/mg protein) vs the concentration of zinc used for induction (first row under the horizontal axis), the fold-induction relative to HeLa cells (second row), and the fold-induction relative to HeLa(Mt-ORF6) cells maintained in the absence of zinc (third row), for the HeLa(MT-ORF6) cells transduced at an MOI of 1,000 AV.CMVLacZ virus particles/cell in the absence of zinc sulfate inducer or in the presence of 50, 100, 150, 200 or 250 μ M zinc sulfate inducer.

Fig. 10 is a schematic of the plasmid pAV.CMVLacZ [SEQ ID NO: 4].

Fig. 11 illustrates plasmid pAV.CMVALP.GRE-ORF6 [SEQ ID NO: 5].

Detailed Description of the Invention

The present invention provides packaging cell lines, which enable the production of recombinant adenoviruses (rAd) functionally deleted in both the E1 and E4 genes. These rAd and methods which enable the therapeutic treatment of disorders with such rAds are disclosed. Novel "second generation" recombinant adeno-associated virus (rAAV) and methods for enhancing the transduction efficiency of rAAV containing a transgene for expression in a somatic gene therapy protocol are also provided. The methods and compositions of this invention are useful in *ex vivo* applications of gene therapy, such as in the transduction of bone marrow cells with desirable hematopoietic stem cell progenitor genes prior to bone marrow transplantation. The embodiments of the invention

are also useful in pharmaceutical compositions for direct in vivo treatment of patients by gene therapy vectors, including the transduction of desirable genes in patients with genetic disorders, such as cystic fibrosis.

5 *I. Packaging Cell Lines*

To increase the transgene capacity and decrease immune response of rAds, as many viral genes as possible should be deleted to inactivate the adenovirus. However, it is crucial to generate complementing cell lines for 10 construction and propagation of such deleted Ad. The method and compositions of the present invention overcome several problems previously identified in the gene therapy for first generation E1 deleted adenoviruses and display advantages in administration particularly to 15 muscle tissue.

Early region 4 (E4) of Ad serotype 5 consists of 7 ORFs believed to be involved in viral DNA replication, host cell shut-off, and late mRNA accumulation. To generate rAd deleted in E4, the function of the E4 region 20 must be supplied to the rAd by a helper virus or packaging cell line. However, useful packaging cell lines have not been available previously because normally the continuous expression of functioning Ad E1 and functional E4 in a single cell line are toxic to the 25 cell. Such cells are therefore not useful for the growth and replication of rAds. Further, the DNA encoding the functional Ad E1 and Ad E4 genes, when present in a packaging cell line, can increase the chances of recombination with a rAd virus to cause the virus to 30 revert to a wildtype Ad virus.

The present invention avoids these problems by providing a packaging cell line which contains the Ad5 E1 gene and only the ORF 6 of the Ad5 E4 gene. ORF6 of E4 alone can provide the requirements for E4 in the viral 35 life cycle.

According to this invention, the ORF6 is preferably under the transcriptional control of an inducible promoter. The mouse mammary tumor virus (MMTV) promoter, inducible by a glucocorticoid, particularly, 5 dexamethasone, is presently preferred. The DNA sequence of the MMTV promoter spans nucleotides 1-1506 of SEQ ID NO: 1. Another inducible promoter is the sheep metallothioneine (MT) promoter, inducible by zinc [M. G. Peterson et al, Eur. J. Biochem., 174:417-424 (1988)]. 10 However, the zinc sulfate inducer of the MT promoter can itself be toxic to the cells. Other inducible promoters, such as those identified in International patent application WO95/13392, published May 18, 1995, and incorporated by reference herein may also be used in the 15 production of packaging cell lines according to this invention. Constitutive promoters, such as the constitutive Ad5 E4 region promoter, LTR, may be employed in control of the expression of ORF6.

The packaging cell line of the invention which 20 utilizes an inducible promoter permits one to control the development of toxicity by regulating the expression of the E4 ORF6 gene. After the desired shuttle vector containing the Ad sequences is transfected into the cell line, expression of the E4 ORF6 can be induced by the 25 appropriate inducer. The packaging cell is thus able to provide both Ad E1 and Ad E4 ORF6 gene products to the rAd for a sufficient period to allow productive infection and recovery of the rAd, before the cell becomes toxic. At present, the time period before the cell experiences 30 toxicity is about 10 days.

In its most preferred form, the packaging cell line is a human embryonic kidney (HEK) 293 E1 expressing cell line into which is introduced the E4 ORF 6 sequence under the control of the inducible promoter. It should be 35 understood by one of skill in the art that another parent

cell line may be selected for the generation of a novel cell line expressing the E1a, E1b, and E4 ORF6 genes of a selected adenovirus serotype. Among such parent cell lines may be included HeLa [CCL 2], A549 [CCL 185], KB [CCL 17], Detroit [e.g., Detroit 510, CCL 72] and WI-38 [ATCC CCL 75] cells. These cell lines are all available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA. Other suitable parent cell lines may be obtained from other sources. If such parent cell lines were selected for modification, the cell line would need to be further supplied with the E1a and E1b gene functions, e.g., such as by transfection with a plasmid containing these genes or functional fragments thereof under a suitable promoter, as well as with the ORF6 gene as described herein.

Example 1 teaches construction of packaging cell lines containing only the ORF 6 of Ad5 E4 region or, for functional comparisons, the entire E4 region. Briefly described, the entire E4 region and an ORF6 sequence of Ad 5 E4 gene were obtained by known techniques [see, e.g., Sambrook et al., "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989) and references cited therein]. To isolate the ORF6 region, the anchored PCR technique was used to amplify the ORF6 sequence from its initiation codon to its termination codon. Primers selected from the published sequence of ORF6 were used to amplify the ORF sequence and insert restriction sites onto the end of the sequence. The E4 ORF6 sequence itself is reproduced as nucleotides 1523 through 2408 of SEQ ID NO: 1. The entire E4 gene sequence is published in the Genbank sequence of Ad5 [Genbank Accession No. M73260].

A minigene was constructed that placed the ORF6 sequence under the control of a selected promoter. By "minigene" as used here is meant the combination of the

desired sequence to be expressed (in this particular instance, the ORF6 sequence) and the other regulatory elements necessary to transcribe the desired sequence and express the gene product in a cell containing that 5 minigene. The ORF6 sequence gene is operatively linked to regulatory components in a manner which permits its transcription. Such components include conventional regulatory elements, such as a promoter to drive ORF6 expression. One inducible promoter was the Zn⁺² 10 inducible MT promoter; the other was the dexamethasone-inducible MMTV promoter of SEQ ID NO: 1.

The minigene also contains nucleic acid sequences heterologous to the ORF6 viral sequence, including sequences providing signals required for efficient 15 polyadenylation of the transcript (poly-A or pA). A common poly-A sequence which is employed in this invention is that derived from the growth hormone (GH) gene terminator sequence (nuc. 2409-3654 of SEQ ID NO: 1). The poly-A sequence generally is inserted in the 20 minigene following the ORF6 sequence. The polyA sequence employed in the MMTV-ORF6 minigene described in Example 1 [SEQ ID NO: 1] is supplied by the GH gene terminator and an SV40 origin of replication (ori). A similar minigene differing in promoter sequence, polyA sequence and/or 25 SV40 ori can also be designed by one of skill in the art to transfer the E4 ORF6 sequence to a shuttle plasmid. Selection of these and other common vector elements are conventional [see, e.g., Sambrook et al, cited above, and references cited therein] and many such sequences are 30 available from commercial and industrial sources as well as from Genbank.

The ORF6-containing minigene was subcloned into a pBR322-based shuttle plasmid that contained a neomycin resistance gene, resulting in the shuttle vector of Fig. 35 1. Any of the many known bacterial shuttle vectors may

be employed to carry the minigene, providing that the vector contains a reporter gene or selectable marker of which many, e.g., neo, amp or purimycin, are known in the art. It is expected that one of skill in the art can 5 develop other suitable shuttle vectors using other plasmid components which are similarly capable of transferring the ORF6 minigene into the chromosome of a cell transfected with the plasmid.

As further described in Example 1, other shuttle 10 vectors were designed for comparative purposes, which contain the complete or substantially complete Ad5 E4 region under the control of the constitutive retroviral MLV LTR sequence in the presence or absence of the endogenous E4 promoter. The shuttle plasmid carrying the 15 ORF6 minigene (or the entire E4 region) was introduced into HEK 293 cells which express the Ad E1 gene products. Complementing cell lines were generated that express these Ad E4 or ORF6 genes from either their endogenous promoters or heterologous inducible promoters. These 20 cell lines are further characterized by their genetic constitution, E4 protein synthesis, recombinant AAV helper function, relative plaque efficiency of H5dl1004 virus, and growth kinetics of recombinant E1/E4 deleted adenovirus. These characteristics of exemplary E1/E4 25 expressing packaging cell lines are discussed in detail in the following examples.

II. Recombinant Adenovirus

The E1/E4 expressing cell line is useful in 30 constructing E1/E4 deleted rAds which can deliver a suitable gene to mammalian cells and tissues. These rAd are functionally deleted in at least the E1a, E1b and E4 Ad gene regions. By the term "functionally deleted" is meant that a sufficient amount of the gene region is removed or otherwise damaged, e.g., by mutation or 35 modification, so that the gene region is no longer

capable of producing the products of gene expression. If desired, the entire gene region may be removed. In *in vivo* experiments with the rAd grown in the packaging cell lines, the E1/E4 deleted rAd demonstrated utility 5 particularly in transferring a transgene to a muscle cell.

The adenovirus sequences used in the construction of the shuttle vectors, helper viruses, if needed, and rAd, and other components and sequences employed in the 10 construction of the vectors and viruses described herein may be readily obtained from commercial or academic sources based on previously published and described sequences. Viral materials may also be obtained from an individual patient. The viral sequences and vector 15 components may be generated by resort to the teachings and references contained herein, coupled with standard recombinant molecular cloning techniques known and practiced by those skilled in the art. Modifications of existing nucleic acid sequences forming the vectors, 20 including sequence deletions, insertions, and other mutations taught by this specification may be generated using standard techniques. Similarly, the methods employed for the selection of viral sequences useful in a vector, the cloning and construction of the "minigene" 25 and its insertion into a desired viral shuttle vector and the production of a recombinant infectious virus are within the skill in the art given the teachings provided herein.

A. Construction of the Transgene

30 A "minigene" in this context is defined as above, except that the components of this minigene are designed to express the gene product *ex vivo* or *in vivo*. Such components include conventional regulatory elements necessary to drive expression of the transgene in a cell 35 transfected with the rAd. For this minigene, a selected

promoter is operatively linked to the transgene and located, with other regulatory elements, within the selected viral sequences of the recombinant vector. Selection of the promoter is a routine matter and is not 5 a limitation of this invention. Useful promoters may be constitutive promoters or regulated (inducible) promoters, which will enable control of the amount of the transgene to be expressed. For example, a desirable promoter is that of the cytomegalovirus (CMV) immediate 10 early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. Another desirable promoter includes the Rous sarcoma virus LTR promoter/enhancer. Still another promoter/enhancer sequence is the chicken 15 cytoplasmic β -actin (CB) promoter [T. A. Kost et al, Nucl. Acids Res., 11(23):8287 (1983)]. Other suitable promoters may be selected by one of skill in the art.

The minigene may also desirably contain nucleic acid sequences heterologous to the viral vector sequences including poly-A sequences and introns with functional 20 splice donor and acceptor sites, as described above. The poly-A sequence generally is inserted in the minigene following the transgene sequences and before the 3' adenovirus sequences. A minigene of the present invention may also contain an intron, desirably located 25 between the promoter/enhancer sequence and the transgene. Selection of these and other common vector elements are conventional as described above and many such sequences are available from commercial and industrial sources as well as from Genbank.

30 As above stated, the minigene is located in the site of any selected deletion in the rAd. In the E1/E4 deleted rAd H5.001CBLacZ, the transgene is located in the deleted E1 gene region. However, the transgene may be located elsewhere in the adenovirus sequence, as desired.

B. *Production of Recombinant Adenovirus*

Adenovirus sequences useful in this invention may include the DNA sequences of a number of adenovirus types, which are available from Genbank, including type 5 Ad5 [Genbank Accession No. M73260]. The adenovirus sequences may be obtained from any known adenovirus serotype, such as serotypes 2, 3, 4, 7, 12 and 40, and further including any of the presently identified 41 human types [see, e.g., Horwitz, cited above]. Similarly adenoviruses known to infect other animals may also be employed in the vector constructs of this invention. The selection of the adenovirus type is not anticipated to limit the following invention. A variety of adenovirus strains are available from the American Type Culture Collection, Rockville, Maryland, or available by request from a variety of commercial and institutional sources. In the following exemplary embodiment an adenovirus, type 5 (Ad5) is used for convenience.

However, it is desirable to obtain a variety of adenovirus shuttle vectors based on different human adenovirus serotypes. It is anticipated that a library of such plasmids and the resulting rAds would be useful in a therapeutic regimen to evade cellular, and possibly humoral, immunity, and lengthen the duration of transgene expression, as well as improve the success of repeat therapeutic treatments. Additionally the use of various serotypes is believed to produce rAd with different tissue targeting specificities. Additionally, the absence of adenoviral genes E1 and E4 in the rAd of this invention should reduce or eliminate adverse CTL responses which normally cause destruction of rAds deleted of only the E1 gene.

rAds of this invention are recombinant, defective adenoviruses (i.e., E1 deleted) which are also deleted completely or functionally of the E4 gene region.

Functional deletions of E4 gene regions may be assessed by assays of Examples 2 and 3, among other assays. rAds useful in this invention may optionally bear other mutations, e.g., temperature sensitive (ts) mutations in the E2a gene region, and deletions in the E3 gene regions.

An adenovirus of this invention contains a functional deletion of the adenoviral early immediate early gene Ela (which spans mu 1.3 to 4.5) and delayed early gene Elb (which spans mu 4.6 to 11.2). Similarly the adenovirus has a functional deletion of the whole E4 region (which spans mu 92 to 97.2), or of at least ORF6 of the E4 region. Gene regions which may be optionally deleted in the E1/E4 deleted rAd of this invention include all or a portion of the adenovirus delayed early gene E3 (which spans mu 76.6 to 86.2). The function of E3 is irrelevant to the function and production of the rAd.

The rAd of this invention may also have a mutation which results in reduced expression of adenoviral protein and/or reduced viral replication. For example, a ts mutation may be introduced into the adenovirus delayed early gene E2a (which spans mu 67.9 to 61.5). Among such mutations include the incorporation of the missense ts mutation in the (DBP)E2a region found in the Ad5 H5ts125 strain [P. Vander Vliet et al, J. Virol., 15:348-354 (1975)] at 62.5 mu. A single amino acid substitution (62.5 mu) at the carboxy end of the 72 kd protein produced from the E2a gene in this strain produces a protein product which is a ss DNA binding protein and is involved in the replication of adenoviral genomic DNA. At permissive temperatures (approximately 32°C) the ts strain is capable of full life cycle growth on HeLa cells, while at non-permissive temperatures (approximately 38°C) no replication of adenoviral DNA is

seen. In addition, at non-permissive temperatures, decreased immunoreactive 72 kd protein is seen in HeLa cells. See, e.g., J. F. Engelhardt et al, Hum. Gene Ther., 5:1217-1229 (1994); J. F. Engelhardt et al, Proc. Natl. Acad. Sci., USA, 91:6196-6200 (1994) and International patent application WO95/13392, published May 18, 1995, incorporated by reference herein.

However, it should be understood that other deletions in the adenovirus genome as previously described in the art or otherwise may also occur in the rAdS of this invention. One minimal type of rAd can contain adenovirus genomic sequences from which all viral genes are deleted. More specifically, the adenovirus sequences may be only the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as oriS) and the native 5' packaging/enhancer domain, that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter. The adenovirus 5' sequence containing the 5' ITR and packaging/enhancer region (Ad5 mu 0-1 or bp 1-360) can be employed as the 5' adenovirus sequence in rAd of this invention. The 3' adenovirus sequences including the right terminal (3') ITR sequence of the adenoviral genome spanning about bp 35,353 - end of the adenovirus genome, or map units ~98.4-100 may be desirably employed as the 3' sequence of the rAd. These sequences, which are clearly devoid of the E1 and E4 genes, can flank, or be operatively associated with the minigene in a rAd. Any other necessary Ad gene products will then be supplied by helper viruses and the E1/E4 ORF6 expressing packaging cell of this invention.

Exemplary rAd for use in this invention, for example, may be obtained by homologous recombination of desired fragments from various rAd, a technique which has been commonly employed to generate other rAd for gene

therapy use. In the examples below, a representative rAd, H5.001CBLacZ, is constructed by homologous recombination between the adenovirus dl1004 (also H5dl1004) viral backbone and pAdCBLacZ minigene DNA.

5 H5dl1004 is an Ad5 virus deleted of from about map unit 92.1 through map unit 98, i.e., substantially the entire E4 gene. The dl1004 virus is described in Bridge and Ketner, J. Virol., 632(2):631-638 (Feb. 1989).

10 The pAdCBLacZ vector is a cDNA plasmid containing Ad m.u. 0-1, an E1 deletion into which is inserted a bacterial β -galactosidase gene under the control of a chicken β -actin promoter, with other regulatory elements as described below, and flanked by Ad m.u. 9-16 and plasmid sequence.

15 The production of the E1/E4 rAd of this invention in the packaging cell line of this invention utilizes conventional techniques. Such techniques include conventional cloning techniques of cDNA such as those described in texts [Sambrook et al, cited above],
20 use of overlapping oligonucleotide sequences of the adenovirus genomes, PCR and any suitable method which provides the desired nucleotide sequence. Standard transfection and co-transfection techniques are employed, e.g., CaPO₄ transfection techniques using the
25 complementation 293 cell line. Other conventional methods employed include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

30 For example, following the construction and assembly of the desired minigene-containing plasmid vector pAdCBLacZ, the E1/E4 expressing packaging cell line of this invention is infected with the helper virus H5dl1004. The infected cell line is then subsequently transfected with an adenovirus plasmid vector by
35 conventional methods. Homologous recombination occurs

between the E4-d 1 t d H5dl1004 helper and the pAdCBLacZ vector, which permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the rAd. About 30 or more hours 5 post-transfection, the cells are harvested, an extract prepared and the rAd containing the LacZ transgene is purified by buoyant density ultracentrifugation in a CsCl gradient.

III. Use of the Recombinant Virus in Gene Therapy

10 The rAd containing the transgene produced by cooperation of the adenovirus vector and E4 deleted helper virus and packaging cell line, as described above, provides an efficient gene transfer vehicle which can deliver the transgene in a pharmaceutical composition to 15 a patient *in vivo* or *ex vivo* and provide for integration of the gene into a mammalian cell.

20 The rAd are administered to humans in a conventional manner for gene therapy and serve as an alternative or supplemental gene therapy for the disorder to which the transgene is directed. A rAd of this invention may be administered to a patient, preferably suspended in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic 25 sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to those of skill in the art may be employed for this purpose.

30 The rAd are administered in sufficient amounts to transfect the desired target cells, e.g., muscle, liver, epithelial, etc. and provide sufficient levels of transfer and expression of the transgene to provide a therapeutic benefit without undue adverse or with 35 medically acceptable physiological effects which can be determined by those skilled in the medical arts.

Conventional and pharmaceutically acceptable routes of administration include direct delivery to the muscle or other selected cell, intranasal, intravenous, intramuscular, subcutaneous, intradermal, oral and other 5 parental routes of administration. Routes of administration may be combined, if desired.

Dosages of rAd will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. 10 For example, a therapeutically effective human dose of the rAd is generally in the range of from about 20 to about 100 ml of saline solution containing concentrations of from about 1×10^9 to 1×10^{11} pfu/ml virus. A preferred human dose is estimated to be about 50 ml saline solution 15 at 2×10^{10} pfu/ml. The dose will be adjusted to balance the therapeutic benefit against any side effects. The levels of expression of the transgene can be monitored to determine the frequency of administration.

An optional method step involves the co- 20 administration to the patient, either concurrently with, or before or after administration of the rAd of a suitable amount of a short acting immune modulator. The selected immune modulator is defined herein as an agent capable of inhibiting the formation of neutralizing 25 antibodies directed against the recombinant vector of this invention or capable of inhibiting or substantially delaying cytolytic T lymphocyte (CTL) elimination of the vector. Among desirable immune modulators are interleukin-12 [European Patent Application No. 441,900]; 30 gamma interferon [S. C. Morris et al, J. Immunol., 152:1047 (1994)]; interleukin-4 [United States Patent No. 5,017,691]; antibody to the CD4 protein, such as anti-OKT 35 3+ [see, e.g., US Patent No. 4,658,019] or antibody GK1.5 (ATCC Accession No. TIB207); a soluble CD40 molecule or an antibody to CD40 ligand (Bristol-Myers Squibb Co)

[European patent application 555,880, published August 18, 1993]; a soluble form of B7 or an antibody to CD28 or CTLA4 [CTLA4-Ig (Bristol-Myers Squibb Co), European patent application 606,217, published July 20, 1994], or 5 agents such as cyclosporin A or cyclophosphamide. Thus, the pharmaceutical compositions and methods of this invention provide a desirable gene therapy treatment.

IV. Recombinant Adeno-Associated Virus

In the following context the term "transgene" means 10 a nucleic acid sequence or reverse transcript thereof, heterologous to the AAV sequence, which encodes a polypeptide or protein of interest. The transgene may be operatively linked to regulatory components in a manner which permits transgene transcription, i.e., the 15 transgene is placed into operative association with a promoter, as well as other regulatory sequences, such as SV40 introns or polyA sequences, useful for its regulation. The composite association of the transgene with its regulatory sequences is referred to herein as a 20 minicassette or minigene.

The composition of the transgene or minicassette sequence will depend upon the use to which the resulting rAAV will be put. For example, one type of transgene sequence includes a reporter sequence, which upon 25 expression produces a detectable signal. Such reporter sequences include without limitation, an *E. coli* β -galactosidase (*LacZ*) cDNA, an alkaline phosphatase gene (ALP) and a green fluorescent protein gene. These sequences, when associated with regulatory elements which 30 drive their expression, provide signals detectable by conventional means, e.g., ultraviolet wavelength absorbance, visible color change, etc.

Another type of transgene sequence includes a therapeutic gene which expresses a desired gene product 35 in a host cell. These therapeutic nucleic acid sequences

typically encode products for administration and expression in a patient *in vivo* or *ex vivo* to replace or correct an inherited or non-inherited genetic defect or treat an epigenetic disorder or disease. Such transgenes 5 may be readily selected by one of skill in this art and the design of the transgene or the minicassette for insertion into the rAAV is not a limitation of this invention.

The term "rAAV" encompasses any recombinant AAV gene 10 therapy vehicle of the prior art, including the AdAAV hybrid virus described in published International Patent Application No. WO96/13598, published May 9, 1996. More specifically, rAAV defines a rAAV comprising: (a) the DNA of at least a portion of the genome of an AAV, which 15 portion is capable of transducing into a target cell at least one selected gene in the absence of cell division; and (b) at least one selected gene (or transgene) operatively linked to regulatory sequences directing its 20 expression, the gene flanked by the DNA of (a) and capable of expression in the target cell *in vivo* or *in vitro*.

Other rAAVs have been described in the art. The method of this invention is not limited by the precise nature of the AAV sequences used in the rAAV, provided 25 that at a minimum both the 5' and 3' AAV inverted terminal repeats are present. Thus, the rAAV may be selected by one of skill in the art, and is not itself a limitation on this invention. The rAAVs specifically disclosed herein are illustrative.

30 By the term "transduction" is meant that the rAAV produced by practice of the invention is capable of infecting a desired target cell and expressing the transgene in the cell by harnessing the cell's machinery. Transduction may include stably integrating the viral DNA 35 into a chromosome of the target cell. "Enhanced

transduction" is defined as the ability of the rAAV in the presence of a conversion agent to transduce the target cell, either *in vitro*, *ex vivo* or *in vivo*, at an efficiency greater than a typical prior art rAAV produced in, and purified from, a culture co-infected with an adenovirus or herpesvirus helper.

This method is based on the observation that the limiting step in rAAV mediated transduction of cells for gene therapy is not the internalization or transfer of the ss viral genome, but rather the subsequent conversion of the single-stranded (ss) viral genome to a transcriptionally active double-stranded (ds) form. Formation of ds DNA intermediates is necessary for recombinant gene expression, which is likely to be modulated by viral and cellular factors through posttranscriptional mechanisms. The inventors have designed a method to overcome this rate-limiting step, thereby enhancing transduction ability of an rAAV and ultimately the use of rAAV in gene therapy protocols.

This method of the present invention may employ a conventionally prepared ss rAAV containing a transgene. The prior art produces ss rAAV by co-infection in culture with a helper adenovirus or herpesvirus, followed by purifying the rAAV from the culture contaminants including the helper virus, and infecting the target cell with the rAAV alone. The present invention provides for infecting a target cell with a ss rAAV. However, once the target cell is infected, the infected cell is contacted with an agent which facilitates the conversion of the ss rAAV to the ds form of rAAV. The action of this "facilitating agent" or "conversion agent" causes the ss to ds conversion to occur in the target cell, resulting in enhanced transduction of the recombinant AAV into the target cell. By facilitating the conversion of ss to ds rAAV in the target cell, the method of this

invention may also result in both transduction and stable chromosomal integration of the rAAV into the chromosome of said host cell.

5 Preferably, for use of this invention the "facilitating or conversion agent" may take several forms.

A. *The Conversion Agent is a Helper Virus*

In one embodiment, the agent is a helper virus and the method includes an additional step of co-10 infecting the target cell with the helper virus. The helper virus useful in this method contains a selected gene which can facilitate the conversion of ss rAAV to ds rAAV. The selected gene may encode a gene product or polypeptide (or a functional fragment of the polypeptide 15 which shares the biological activity of the full-length polypeptide) which enhances the conversion.

Alternatively, the selected gene may express an antisense or ribozyme which functions in the cell to block or inhibit a cellular gene that normally prevents ss to ds 20 conversion of the rAAV. These genes may also be employed in the second generation rAAV described below.

The helper virus is capable of expressing the selected gene product in the target cell in the absence of cell division. The helper virus may be a wild-type or 25 mutant adenovirus. The helper virus may alternatively be a wild-type or mutant herpesvirus. Preferably, for use as facilitating agents, such viruses are mutants deleted of several normal genes so that the helper viruses and/or their expressed gene products will not cause disease in a 30 patient.

For example, a helper adenovirus useful in this invention may express only a gene product of a single adenoviral early gene. Exposure of the ss rAAV to an Ad early gene product is sufficient to substantially enhance 35 the formation of ds rAAV genome with a coordinate

increase in transduction efficiency. The Ad early genes which are useful in producing this effect are E1, E2a, E4 and functional fragments thereof. However, as demonstrated by the examples below, adenovirus 5 substantially enhances recombinant AAV transduction in vitro in a way that is dependent on expression of the E1 and E4 genes of adenovirus and is directly proportional to the appearance of ds replicative forms of rAAV.

One example of a helper virus is an adenovirus 10 deleted of most of its wild-type early genes and which is capable of expressing only its E4 gene or a functional fragment thereof in the target cell. Among such functional fragments is the ORF 6 of the E4 gene. As described below in the examples, experiments in cell 15 lines indicate that the ORF6 of the adenoviral E4 gene locus is sufficient to significantly enhance rAAV transduction. Selective expression of the E4-ORF6 product of adenovirus accomplishes a increase in transduction efficiency similar to, but somewhat 20 attenuated, compared to that produced by exposure to the E1 and E4 gene products in combination. That is, the ORF6 product of E4 is sufficient to enhance the augmentation of rAAV transduction; but this effect is amplified substantially by E1 gene products.

25 Thus, more preferably, exposure of the rAAV to both the expressed E1 and E4 gene products produces a substantial enhancement of the above-described rate limiting step. Therefore, another exemplary helper virus may also contain more than one gene which, upon 30 expression, facilitates the ss to ds conversion. An example of such a helper virus is an adenovirus which expresses both the E1 and E4 genes, or functional fragments thereof. Still other Ad genes may be expressed by the helper virus, provided that the virus is 35 sufficiently crippled so that it does not cause disease

in the patient contributing the target cells.

Where the agent which facilitates conversion of ss to ds rAAV is a helper virus, the method of the invention comprises co-infecting the target cell with the 5 rAAV and the helper virus. Such co-infection may occur in the context of *ex vivo* therapy, i.e., manipulations performed on cells extracted from the patient, which cells are reinserted into the patient after the method is performed. Alternatively, the patient may be directly 10 co-infected with the two viruses by conventional means. Delivery of the two viruses to the patient may be directed to a specific organ, or to the general circulatory system. Such delivery methods are described in the art for gene therapy of, e.g., cystic fibrosis 15 [see, e.g., US Patent No. 5,240,846].

B. The Conversion Agent is a Chemical, Drug or Other Entity that can Activate rAAV Transduction

In another embodiment of the method of this invention, the conversion agent which contacts the cells 20 infected with the rAAV may be selected from the following classes of known compounds or methods: 1) inhibitors of DNA synthesis such as hydroxyurea, hydrogen peroxide, and other direct or indirect inhibitors of DNA polymerase; 2) chemo-therapeutic agents that induce DNA damage, such as 25 cyclophosphamide, alkylating agents, purine analogs, e.g., 6-thioguanine, etc.; 3) drugs that interfere with DNA modifying enzymes, such as inhibitors of topoisomerase, DNA ligase exonucleases and endonucleases; and 4) agents that nonspecifically enhance transcription, 30 such as sodium butyrate, or agents that stabilize cells, such as DMSO. Also, genotoxic agents such as carcinogens may be employed as the conversion agent. Other methods of inducing disruption or damage to DNA may also be 35 useful as agents capable of facilitating ss to ds conversion of rAAV and maybe selected by one of skill in

the art, including physical methods, such as irradiation. These classes of compounds or methods are believed to result in the conversion from ss to ds rAAV.

According to this embodiment of the method of the invention, the rAAV is again produced conventionally, but not co-infected with a helper virus. The ss rAAV is infected into the target cell, and the infected cell is contacted by the agent in an appropriate manner depending on the identity of the agent. These conversion enhancing agents can be employed in *ex vivo* treatment of the target cells infected by the rAAV by application directly to the cells. Such application can occur substantially simultaneously, or consecutively, with application of the rAAV gene therapy vehicle. For example, the infected target cell may be subjected to one of the above-listed compounds or drugs for a desired time period. The parameters for contacting the infected cells with the agent may readily be determined by one of skill in the art. These parameters will depend upon whether the method is performed *ex vivo* or *in vivo*. For example, the number of *ex vivo* infected cells to be treated will be considered for the dosage, and timing of such treatment.

Similarly, the physical status of the patient can determine the parameters of delivery of the agent to the patient *in vivo*. The dosage and amount of the damaging agent may therefore be adjusted by one of skill in the art. Where the agents are typical chemotherapeutic drugs approved for use in humans or animals, such enhanced conversion of rAAV may also occur *in vivo* by the co-administration of the agent, i.e., the chemotherapeutic drug, and the rAAV gene therapy vehicle to the patient. According to this aspect of the invention, the chemotherapeutic drug would be administered only when the rAAV is administered. Appropriate dosages and amounts of chemotherapeutic drugs

and recombinant gene therapy vehicles and amounts for determining such amounts are within the skill of the art. However, because the effect of the chemotherapeutic drug will enhance the ss to ds conversion of the rAAV and thus 5 enhance its efficiency of transduction into the target cells, it is anticipated that lower dosages than the conventional dosages of either or both the drug and the rAAV could be effectively administered.

C. Conversion Agent May be Part of the rAAV.

10 In still another embodiment of this invention, a novel "second generation" rAAV may be designed to incorporate the conversion agent into the virus, so that both the transgene and the conversion agent are co-expressed in the target cell. Such a novel recombinant 15 adeno-associated virus comprises the following components:

(a) the DNA of at least a portion of the genome of an adeno-associated virus which portion is capable of transducing at least two selected genes or 20 functional fragments thereof into a target cell in the absence of cell division; (b) a first selected gene, i.e., the desired transgene, operatively linked to regulatory sequences directing its expression, and (c) a second selected gene, i.e., the "conversion gene" 25 operatively linked to regulatory sequences capable of directing expression of said second gene. The "conversion gene" upon expression is capable of facilitating the conversion of the ss rAAV to its ds form upon expression. The first and second genes in this rAAV 30 are flanked by the AAV DNA, preferably the 5' and 3' ITRs. An embodiment of such a second generation rAAV is provided schematically in Fig. 11. Its DNA sequence is provided in SEQ ID NO: 5.

Another embodiment of such a novel rAAV may 35 include more than one gene which upon expression has the

ability to facilitate conversion of ss to ds rAAV in the target cell. For example, the novel rAAV described above may also contain an additional selected gene operatively linked to regulatory sequences capable of directing its expression, the additional gene and said second "conversion" gene described above being capable of jointly facilitating the conversion of ss rAAV to its ds form upon expression of both the second and additional genes. In this rAAV, all three genes, i.e., the transgene, the second "conversion" gene and the additional gene are flanked by the AAV DNA.

In one desirable embodiment of a novel rAAVm the AAV ITRs flank a selected transgene, and a conversion gene, which is the adenovirus E4 gene or a functional fragment thereof (e.g., the ORF6 sequence). In another embodiment, the novel recombinant expresses three genes, the transgene, the adenovirus E4 gene or a functional fragment thereof and the adenovirus E1 gene or a functional fragment thereof. The E4 and E1 gene products expressed in the target cell with the transgene, together act to facilitate conversion of the ss to ds form of rAAV.

In still another embodiment of the novel rAAV and its use, the regulatory sequences directing expression of the conversion gene, e.g., whether it be a single second gene or more than a single additional gene, may include an inducible promoter. Thus, expression of the conversion gene occurs only in the presence of an inducing agent. Many inducible promoters and companion inducing agents, e.g., steroids such as glucocorticoids, are known to the art and may be readily selected for incorporation into the rAAV and methods of this invention by one of skill in the art with resort to this description.

35 The method of the invention employing such

"second generation" rAAVs which carry at least one "conversion gene" provides for infecting the target cell with this ss rAAV. Where the promoters directing expression of both the transgene and the conversion gene 5 are constitutive, the infected target cell machinery will direct the expression of the transgene product and conversion gene product. Co-expression in the target cell of the transgene and the "conversion gene" facilitates the conversion of ss rAAV to ds rAAV in the 10 cell, and increases the transduction efficiency, and perhaps stable chromosomal integration, without further method steps.

When the second generation rAAV employed in the method contains the "conversion gene(s)" under the 15 control of inducible promoter(s), the method is slightly altered. Following infection of the target cell by the rAAV, the infected target cell is contacted with a suitable inducing agent, which triggers the inducible promoter to "turn on" production of the conversion gene 20 product. When the inducing agent is removed or stopped, the expression of the conversion gene product is "turned off".

As described above, any prior art rAAV containing a transgene for gene therapy may be used in at 25 least one embodiment of the above methods. The sources, selection and assembly of the various components to generate the rAAV, including the novel rAAV described above, are now conventional and readily accessible to one of skill in this art, given the disclosure contained 30 herein. Such methods employ conventional genetic engineering techniques [See, e.g. Sambrook et al, cited above].

The novel rAAV viruses and the methods of this invention provide efficient gene transfer vehicles for 35 somatic gene therapy and are suitable in pharmaceutical

compositions for *x vivo* applications and *in vivo* use. When rAAV contain a therapeutic gene, e.g., in place of the *LacZ* transgene illustrated in the exemplary rAAV, AV.CMVLacZ, by use of the rAAV and the methods described herein, the therapeutic transgene can be delivered to a patient *in vivo* or *ex vivo* to provide for efficient transduction, and possibly stable integration, of the desired gene into the target cell. Thus, these novel rAAV and the methods described herein can be employed to correct genetic deficiencies or defects. The potential of AAV to efficiently integrate its genome into nondividing cells is currently being exploited in the development of gene therapies based on *ex vivo* transduction of hematopoietic stem cells. *In vivo* application of rAAV is primarily being developed for the treatment of CF where purified stocks of virus are instilled into the airway to transduce the terminally differentiated epithelial cells of conducting airway. The methods and compositions described herein can be used with both types of gene therapy. Another condition suitable for such use includes transduction of the low density lipoprotein (LDL) receptor gene into hepatocytes for the treatment of familial hypercholesterolemia. One of skill in the art can generate any number of rAAV which can be used via the above methods for the treatment of these and other disorders.

For *ex vivo* or for *in vivo* therapy, the rAAV may be used to infect the target cells by suspending the virus particles in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to

those of skill in the art may be employed for this purpose.

The rAAV are administered in sufficient amounts to transfect the desired cells and provide sufficient 5 levels of expression of the selected transgene to provide a therapeutic benefit without undue adverse, or with medically acceptable, physiological effects which can be determined by those skilled in the medical arts. Conventional and pharmaceutically acceptable routes of in 10 vivo administration include direct delivery to the target organ, tissue or site, intranasal, intravenous, intramuscular, subcutaneous, intra^dermal, oral and other parental routes of administration. Routes of administration may be combined, if desired.

15 Dosages of the rAAV for the infecting step of the method will depend primarily on factors such as the therapeutic environment, i.e., ex vivo or in vivo; the condition being treated, the selected gene, the age, weight and health of the patient, and may thus vary among 20 patients. A therapeutically effective dosage of the rAAV for ex vivo treatment will be based upon the multiplicity of infection, which is likely to range from between about 1 to about 10 transducing particles/cell. A therapeutically effective human dosage of the rAAV for in 25 vivo infection according to the present invention is believed to be in the range of from about 20 to about 50 ml of saline solution containing concentrations of from about 1×10^7 to 1×10^{10} transducing viral particles/ml virus. A preferred human dosage is about 20 ml saline 30 solution at the above concentrations. The dosage will be adjusted to balance the therapeutic benefit against any side effects. The levels of expression of the selected gene can be monitored to determine the selection, adjustment or frequency of dosage administration.

Th effective amount of th facilitating agent to be administered is within the skill of the art to determine and will depend upon the identity of the agent. Known dosages of certain of the classes of chemicals and pharmaceuticals described above may be employed in this method to damage the DNA and facilitate ss to ds conversion of the rAAV. Where the agent is a gene expressed by a helper virus, the amounts of infecting virus should be similar to those amounts described above for the rAAV. Of course, where the agent is a gene present in a second generation rAAV, the identical dosages described above for the rAAV will apply.

Several embodiments of the above-described methods of this invention were confirmed in murine models of rAAV mediated gene transfer to both lung and liver. These experiments demonstrated similarly low levels of gene transfer *in vivo* by rAAV, which was increased several orders of magnitude by coinfection with E1 and E4 expressing adenovirus.

In summary, experiments were conducted to demonstrate that adenovirus enhances rAAV transduction in cultured cells. During the production and characterization of a lacZ recombinant AAV generated in 293 cells that were coinfecte~~d~~ with an E1 deleted virus, it was observed that purification of rAAV from lysates was associated with substantial loss of lacZ transducing activity when assayed on 293 cells. This drop in rAAV activity was particularly evident in the final step where residual contaminating helper adenovirus was removed by heat inactivation. LacZ transducing activity was recovered by adding adenovirus back to the purified stock of rAAV. These data provided the first indication that adenovirus could substantially enhance the transduction fficiency of rAAV.

As described in Example 10, a series of complementation groups were generated by mixing different adenovirus early gene mutants with purified LacZ rAAV, referred to as AV.CMVLacZ (see Example 2). These defined mixtures of viruses were analyzed for LacZ transduction on HeLa cells (See Examples 12 and 13). An E1 deletion rAd H5.CBALP and the E4 deletion mutant dl1004 provided no significant increase in AV.CMVLacZ transduction (Fig. 5A). However, partial activity could be achieved with E1 and E4 mutants that carried less severe deletions. Both dl110 (E1B-55kDa deleted) and dl1010 (ORF6 deleted) enhanced transduction to levels that approached those of Ad5, ts125, and dl802 in terms of the number of positive blue cells, but total β -galactosidase activity was substantially lower (Fig. 5A). These results implicate early regions E1 and E4 in the augmentation of rAAV transduction.

The experiments described below also demonstrate that the novel rAAV which incorporates as its conversion gene, an Ad gene, such as E4, can increase transduction efficiency of the rAAV in the absence of a helper virus. As described in more detail below in Example 15 below, 293 cells were stably transfected with a genomic fragment of Ad5 spanning E4. This E1/E4 expressing cell line and the parent E1 expressing cell line (293) were infected with rAAV and analyzed for transduction. These experiments demonstrated the significance of the combined expression of E1 and E4(ORF6) in the adenovirus mediated augmentation of rAAV transduction.

In the presence of E1 and E4 expression, rAAV transduction was invariably accompanied by the appearance of ds RF monomers and dimers (Example 14). Importantly, the tight correlation between rAAV vector transduction and the accumulation of duplex forms could be achieved in

two different experimental settings; cells infected with E1/E4 expressing adenovirus (Figs. 8A and 8B), or complementing cell lines (Fig. 8C).

The following examples illustrate the 5 construction and testing of the novel packaging cell lines, the E1/E4 deleted rAd of the present invention and the use thereof, improved methods and second generation recombinant AAV production for gene therapy of the present invention. These examples are illustrative only, 10 and do not limit the scope of the present invention.

Example 1 - Novel E1a/E1b and E4 Expressing Packaging Cell Lines

A. Construction of E4 ORF 6 Expressing Plasmids

The entire E4 region from Ad5 or an ORF6 15 minigene were subcloned into a shuttle plasmid that contained a neomycin resistance gene. Two versions of ORF6 minigene were developed that differed in the promoter element. The first used a Zn²⁺ inducible sheep metallothioneine (MT) promoter to drive ORF 6 expression. 20 The second used a dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter.

An exemplary plasmid useful for the construction of a packaging cell line of this invention is pMMTVE4ORF6. The minigene contained in this plasmid 25 is set out in SEQ ID NO: 1, and contains a mouse mammary tumor virus promoter (MMTV) (nucleotides 1-1506 of SEQ ID NO:1) in transcriptional control of a human E4 ORF 6 gene sequence (nucleotides 1523-2408 of SEQ ID NO: 1), a growth hormone terminator (GH) (nucleotides 2409-3654 of 30 SEQ ID NO: 1), an SV40 origin of replication, plasmid sequences from plasmid pBR322, including a neomycin resistance gene, and an ampicillin resistance gene. The amino acid sequence of ORF 6 is indicated in SEQ ID NO: 2. The various functional fragments of this plasmid may 35 be readily replaced with other conventionally used

s quences and are n t critical to the design of the plasmid.

Another plasmid useful for the construction of a packaging cell line of this invention is pMTE4ORF6.

5 The DNA sequence of the minigene contained in this plasmid is similar to that of SEQ ID NO: 1, except that the promoter is a sheep metallothionein promoter (MT promoter) [M. G. Peterson et al, cited above].

10 A plasmid used as a control for the construction of a packaging cell line of this invention is pLTR.E4(-). This plasmid contains the endogenous constitutive retroviral MLV LTR and most of the Ad E4 gene region except that the endogenous E4 promoter and a portion of E4 ORF1 are missing. The other plasmid sequences remain the same as described above.

15 Still another plasmid useful for the study of the methods of this invention is pLTR.E4, which contains the constitutive MLV LTR and endogenous E4 promoter and an intact E4 gene. The other plasmid sequences remain the same as described above.

20 To determine whether ORF6 expression was sufficient to enhance rAAV transduction, the inducible metallothionein (MT)-ORF6 minigene was stably transfected into HeLa cells. This new cell line, HeLa(MT-ORF6) was evaluated for LacZ rAAV transduction in response to ORF6 induction as described below. The cell line 293 (MT-ORF6) expresses ORF-6 of the E4 gene of Ad5 from the metallothionein promoter which is relatively inactive at baseline but can be induced with divalent cations. These 293 cells were included to establish the baseline transduction efficiency.

25 **B. Transfections and Selection of Clones**

30 Each of the above-described plasmids was transfected by the calcium phosphate precipitati n technique into the human embryonic kidney cell line 293

[ATCC CRL1573] which expresses the product of the adenovirus E1 genes, or into HeLa cells, seeded on 100 mm plates (10 µg plasmid/plate). Twenty four hours post-transfection, cells were harvested and seeded at varying 5 dilutions (1:10 - 1:100) in 100 mm plates for about 10 days. Seeding media contain G418 (Geneticin, BRL) at 1 mg/ml. Resistant colonies that developed were selected using the following assays and expanded. Preliminary analysis of clones was based on enhanced transduction 10 efficiency of a recombinant adeno-associated virus, AV.CMVLacZ, and immunofluorescence localization of Ad E4 protein as described in the following examples.

Example 2 - Recombinant AAV and AV.CMVLacZ Transduction Enhancement Assay

15 E1 and E4 Ad gene products are needed for recombinant adeno-associated virus (AAV) function. This primary assay involves seeding the packaging cell lines of Example 1 in 96 well 35 mm culture plates (2x10⁶ cells/well) and infecting the cells with purified, heat- 20 treated AV.CMVLacZ at an MOI of 1000 virus particles/cell.

A. *Preparation of Recombinant AV.CMVLacZ*

A recombinant AAV virus was prepared by conventional genetic engineering techniques for the 25 purposes of this experiment. Recombinant AAV was generated by plasmid transfections in the presence of helper adenovirus [Samulski et al, *J. Virol.*, 63:3822-3828 (1989)]. A cis-acting plasmid pAV.CMVLacZ [SEQ ID NO: 4] (see Fig. 10) was derived from psub201 [Samulski 30 et al, *J. Virol.*, 61:3096-3101 (1987)] and contains an *E. coli* β-galactosidase minigene in place of AAV Rep and Cap genes. The 5' to 3' organization of the recombinant AV.CMVLacZ genome (4.9 kb) [SEQ ID NO: 4] includes (a) the 5' AAV ITR (bp 1-173) was 35 obtained by PCR using pAV2 [C. A. Laughlin et al, *Gene*,

23: 65-73 (1983)] as template [nuc. 53-219];
(b) a CMV immediate early
enhancer/promoter [Boshart et al, Cell, 41:521-530
(1985)] (nuc. 246-839);
5 (c) an SV40 intron (nuc. 856-987);
(d) E. coli B-galactosidase cDNA (nuc.
1039-4512);
(e) an SV40 polyadenylation signal (a 237
Bam HI-BclI restriction fragment containing the
10 cleavage/poly-A signals from both the early and late
transcription units (nuc. 4522-4719) and
(f) 3'AAV ITR, obtained from pAV2 as a
SnaBI-BglII fragment (nuc. 4759-4925). All other
nucleotides are plasmid derived.

15 Rep and Cap genes were provided by a trans-
acting plasmid pAAV/Ad [Samulski et al, cited above].

Monolayers of 293 cells grown to 90% confluency
in 150 mm culture dishes (5×10^7 cells/plate) were
infected with H5.CBALP at an MOI of 10. H5.CBALP (also
20 called H5.010ALP) is a rAd that contains an alkaline
phosphatase minigene in place of adenovirus E1a and E1b
gene sequences (map units 1-9.2 of the Ad5 sequence of
GenBank [Accession No. M73260]). The alkaline
phosphatase cDNA is under the transcriptional control of
25 a CMV-enhanced β -actin promoter in this virus. This
helper virus is described in Goldman et al, Hum. Gene
Ther., 6:839-851 (July, 1995); Engelhardt et al, Hum.
Gene Ther., 5:1217-1229 (October, 1994); and references
cited therein.

30 Infections were done in Dulbecco's Modified
Eagles Media (DMEM) supplemented with 2% fetal bovine
serum (FBS) at 20 ml media/150 mm plate. Two hours post-
infection, 50 μ g plasmid DNA (37.5 μ g trans-acting and
12.5 μ g cis-acting) in 2.5 ml of transfection cocktail
35 was added to each plate and evenly distributed.

Transfections were calcium phosphate based as described [B. Cullen, Meth. Enzymol., 152:684-704 (1987)]. Cells were left in this condition for 10-14 hours after which the infection/transfection media was replaced with 20 ml
5 fresh DMEM/2% FBS. Forty to fifty hours post-transfection, cells were harvested, suspended in 10 mM Tris-Cl (pH 8.0) buffer (0.5 ml/150 mm plate) and a lysate prepared by sonication. The lysate was brought to 10 mM manganese chloride, after which bovine pancreatic
10 DNase I (20,000 units) and RNase (0.2 mg/ml final concentration) were added, and the reaction incubated at 37°C for 30 minutes. Sodium deoxycholate was added to a final concentration of 1% and incubated at 37°C for an additional 10 minutes.

15 The treated lysate was chilled on ice for 10 minutes and solid CsCl added to a final density of 1.3 g/ml. The lysate was brought to a final volume of 60 ml with 1.3 g/ml CsCl solution in 10 mM Tris-Cl (pH 8.0) and divided into three equal aliquots. Each 20 ml sample was
20 layered onto a CsCl step gradient composed of two 9.0 ml tiers with densities 1.45 g/ml and 1.60 g/ml.

25 Centrifugation was performed at 25,000 rpm in a Beckman SW-28 rotor for 24 hours at 4°C. One ml fractions were collected from the bottom of the tube and analyzed on 293 or 293(E4) cells for LacZ transduction. Fractions containing peak titers of functional AV.CMVLacZ virus were combined and subjected to three sequential rounds of equilibrium sedimentation in CsCl. Rotor selection included a Beckman NVT-90 (80,000 rpm for 4
30 hours) and SW-41 (35,000 rpm for 20 hours). At equilibrium, AV.CMVLacZ appeared as an opalescent band at 1.40-1.41 g/ml CsCl. Densities were calculated from refractive index measurements. Purified vector was exchanged to 20 mM HEPES buffer (pH7.8) containing 150 mM
35 NaCl (HBS) by dialysis and stored frozen at -80°C in the

presence of 10% glycerol or as a liquid stock at -20°C in HBS/40% glycerol.

Purified virus was tested for contaminating H5.CBALP helper virus and AV.CMVLacZ titers. Helper 5 virus was monitored by histochemical staining for reporter alkaline phosphatase activity. A sample of purified virus representing 1.0% of the final product was added to a growing monolayer of 293 cells seeded in a 60 mm plate. Forty-eight hours later, cells were fixed in 10 0.5% glutaraldehyde/phosphate buffered saline (PBS) for 10 minutes at room temperature, washed in PBS (3x10 minutes) and incubated at 65°C for 40 minutes to 15 inactivate endogenous alkaline phosphatase activity. The monolayer was allowed to cool to room temperature, rinsed once briefly in 100 mM Tris-Cl (pH 9.5)/100 mM NaCl/5 mM MgCl₂, and incubated at 37°C for 30 minutes in the same buffer containing 0.33 mg/ml nitroblue tetrazolium chloride (NBT) and 0.165 mg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP). Color 20 development was stopped by washing the monolayer in 10 mM Tris-Cl (pH 8.0)/5 mM EDTA. Routinely the purification scheme described above removed all detectable H5.CBALP helper virus by the third round of buoyant density ultracentrifugation.

AV.CMVLacZ titers were measured according to 25 genome copy number (virus particles/ml), absorbance at 260 nm (A₂₆₀ particles/ml) and LacZ Forming Units (LFU/ml). Virus particle concentrations were based on Southern blotting. Briefly, a sample of purified 30 AV.CMVLacZ was treated with capsid digestion buffer (50 mM Tris-Cl, pH 8.0/1.0 mM EDTA, pH 8.0/0.5% SDS/Proteinase K 1.0 mg/ml) at 50°C for one hour to release virus DNA. The reactions were allowed to cool to room temperature, loading dye was added and 35 electrophoresed through a 1.2% agarose gel. Standard

quantities of ds AV.CMVLacZ genome were also resolved on the gel.

DNAs were electroblotted onto a nylon membrane, hybridized with a ³²P random primer labeled restriction fragment, and the resulting blot scanned on a PhosphorImager 445 SI (Molecular Dynamics). A standard curve was generated from the duplex forms and used to extrapolate the number of virus genomes in the sample. LFU titers were generated by infecting indicator cells with limiting dilutions of virus sample. Indicator cells included HeLa and 293 and 293 (E4) lines (described in Example 10 below). Twenty-four hours later, cells were fixed in glutaraldehyde and cells were histochemically stained for *E. coli* β -galactosidase (LacZ) activity as described in J. M. Wilson et al, Proc. Natl. Acad. Sci. USA, 85:3014-3018 (1988). One LFU is described as the quantity of virus that is sufficient to cause visually detectable β -galactosidase expression in one cell 24 hours post-infection.

20 B. *Induction of ORF6 Expression*

Induction of ORF6 expression with 10 μ M dexamethasone or 150 μ M zinc sulfate (for negative control, no inducer used) was initiated 2 hours before the addition of virus and continued throughout the duration of the experiment. Twenty-four hours after the addition of virus, cells were harvested, lysates were generated by sonication and analyzed for the β -galactosidase expression (i.e., β -galactosidase activity) and virus DNA as described above. Hirt extracts were prepared from low molecular weight DNA from cell extracts. The preparation of the Hirt extracts and subsequent analysis by Southern hybridization were performed by resort to conventional procedures known to one of skill in the art.

35 In the absence of the inducers, the packaging

5 cell lines generate lower levels of β -galactosidase in rAAV infected cells. Induction of ORF6 expression with the inducer dexamethasone results in a concomitant rise in AV.CMVLacZ cell transduction to a level that was much greater than the parent 293 line. Expression of E1 alone was insufficient to have an effect in the adenovirus mediated augmentation of rAAV transduction.

10 Results are demonstrated for certain positive clones in the Table I below (see Example 4). However, for 30 cell lines having an MMTV promoter and ORF6 sequence, 4 demonstrated over 90% blue cells illustrative of LacZ production in the presence of dexamethasone, i.e., 293-27-6, 293-27-17, 293-27-18 and 293-27-28.

15 Example 3 - Immunofluorescence Localization of Ad5 Late Protein.

Positive clones from the assay of Example 2 were infected with the recombinant E4 deleted adenovirus H5dl1004 and screened for E4 complementation using an immunofluorescence assay for late gene expression. The 20 H5dl1004 virus was obtained from Dr. Ketner of Johns Hopkins University and is described in Bridge and Ketner, J. Virol., 632(2):631-638 (Feb. 1989), incorporated by reference herein. Because ORF6 of E4 complements late Ad gene expression, specifically in the formation of the 25 hexon and penton fibers of the adenovirus, cell lines containing ORF6 are able to bind with antibody against these proteins.

Each cell line of Example 1 is infected with E4 deleted virus H5dl1004 virus at an MOI of 0.1. The cells 30 were treated with mouse anti-adenovirus FITC-labeled monoclonal antibody to either the hexon or penton fibers in a 1:10 dilution (Chemicon International Inc., Temecula, CA). Positive clones were identified by reaction with the antibody.

Example 4 - Relative Plaquing Efficiency

The cell lines of Example 1 demonstrating with strong complementation ability in Example 3 were screened for relative plaquing efficiency of H5dl1004 as compared 5 to W162 cells (an E4-complementing Vero cell line which does not express E1) [Weinberg and Ketner, Proc. Natl. Acad. Sci. USA, 80(17):5383-5386 (1983)]. In Table II below, RPE%, i.e., relative plaquing efficiency, represents the titer of H5dl1004 on tested cell 10 lines/titer of H5dl1004 on W162 cells. For example, the RPE of 293 cells is 0.

The positive cell lines selected by all criteria are identified in Table I below, with the results of the assays of Examples 2, 3 and 4.

15

TABLE I

E1/E4 Double Complementing Cell Lines

	<u>Cell Line</u>	<u>Trans- Gene</u>	<u>Pro- moter</u>	<u>IF/LP</u>	<u>AV. CMV LacZ</u>	<u>RPE%</u>
	293-10-3	ORF6	MT	++++	++++	246
20	293-39-11	ORF6	LTR	++++	+++	52
	293-84-31	E4-	LTR	++++	++++	179
	293-12-31	whole E4	LTR +E4	++++	++++	174
	293-27-6	ORF6	MMTV		+++++	327
25	293-27-17	ORF6	MMTV		++++	313
	293-27-18	ORF6	MMTV		+++++	339
	293-27-28	ORF6	MMTV		++++	261

Example 5 - Construction and Purification of H5.001CBLacZ

30 The plasmid pAd.CBLacZ was constructed as described in detail in K. Kozarsky et al, Som. Cell Mol. Genet., 19(5): 449-458 (1993), incorporated by reference herein. This plasmid contained a minigene comprising a 5'

flanking NheI restriction site, followed by Ad5 sequence m.u. 0-1, followed by an E1 deletion into which is inserted a CMV enhancer/chicken β -actin promoter sequence [T. A. Kost et al, *Nucl. Acids Res.*, 11(23):8287 (1983)], 5 which controls the transcription of the following bacterial β -galactosidase, followed by a poly A sequence and flanked 3' by Ad m.u. 9-16, and another NheI site. In the plasmid, the minigene was flanked on both sides by plasmid sequence containing drug resistance markers.

10 The plasmid pAd.CBLacZ was linearized with NheI and co-transfected by the calcium phosphate co-transfection method into the novel packaging cell line of Example 1 with ClaI digested H5dl1004 (an Ad5 sequence deleted of from about map unit 92.1 through map unit 98, 15 corresponding to substantially the entire E4 gene).

Homologous recombination occurs in the cell line between these two viral constructs between Ad map units 9-16, resulting in rAd, designated H5.001CBLacZ [SEQ ID NO: 3] (Fig. 2). This rAd contains the sequence from 20 pAd.CBLacZ (including Ad map units 0-1 (nuc. 1-330); CMV enhancer/chicken β -actin promoter (CB) (nucs. 370-928); *E. coli* β -galactosidase (nucs. 945-4429); the polyA (nuc. 4429-4628); and Ad5 map units 9-92.1 and 97.3 to 100 from H5dl1004 (nucs. 4671-35408)). This rAd is thereby 25 functionally deleted, and substantially structurally deleted, of the Ad E1 and E4 genes.

Viral plaques were selected and screened by the β -galactosidase assay [Wilson (1988), cited above] and H5.001CBLacZ was isolated following three rounds of 30 plaque purification. The purified virus was also subjected to cesium chloride density centrifugation and large scale production.

For the following mouse experiments, virus was used after column purification and glycerol was added to a

final concentration of 10% (v/v). Virus was stored at -70°C until use.

Example 6 - Growth Kinetics of H5.001CBLacZ in Packaging Cell Lines

5 The cell lines identified in Table I were infected with recombinant H5.001CBLacZ at an MOI of .5. The growth kinetics of this virus in the E4 complementing cell lines are shown in Fig. 3. Maximum viral yield is reported as LFU/ml in Table II below.

10

TABLE II

	<u>Cell Line</u>	<u>Maximum Viral Yield</u>
	293-10-3	2.8×10^{10}
	293-39-11	9.5×10^8
	293-84-31	1.1×10^9
15	293-12-31	4.5×10^8
	293-27-6	2.8×10^{10}
	293-27-17	2.5×10^{10}
	293-27-18	2.9×10^{10}
	293-27-28	1.2×10^{10}

20 When grown in 293-27-18 cells (the E4 ORF6 cell line with MMTV promoter inducible by dexamethasone) the maximum yield of this virus is 2.9×10^{10} LFU/ml. Several of the cell lines were passaged between 5 and 20 times and the viral production of the passages remained stable.

25 However, RPE did fall following repeated passages of cells.

Example 7 - Other Recombinant Adenoviruses

30 Other related rAds were prepared similarly to H5.001CBLacZ by homologous recombination between pAdCBLacZ and other helper viruses.

35 As one example, H5.000CBLacZ is a recombinant E1 deleted Ad5 which contains the same minigene as H5.001CBLacZ, but has an intact E4 gene. This rAd was prepared as described by homologous recombination between pAdCBLacZ and a wild-type Ad5.

As another example, H5.010CBLacZ contains the adenovirus map units 0-1, followed by a CMV enhanced, chicken cytoplasmic β -actin promoter, the *E. coli* β -galactosidase gene (lacZ), a polyadenylation signal (pA), and adenovirus type 5 map units 9-100, with a small deletion in the E3 gene (the Ad 5 sub360 backbone). This rAd may be prepared by homologous recombination between the pAdCBLacZ vector and Ad5 virus sub360, which contains a 150 bp deletion within the 14.6 kD protein of the E3 gene. See, e.g., J. F. Engelhardt et al, Proc. Natl. Acad. Sci. USA, 91:6196-6200 (June 1994); and Engelhardt et al, Hum. Gene Ther., 5:1217-1229 (Oct. 1994), both incorporated by reference herein.

These rAds were isolated following transfection [Graham, Virol., 52:456-467 (1974)], and were subjected to two rounds of plaque purification. Lysates were purified by cesium chloride density centrifugation as previously described [Englehardt et al, Proc. Natl. Acad. Sci. USA, 88:11192-11196 (1991)]. Cesium chloride was removed by passing the virus over a BioRad DG10 column using phosphate-buffered saline.

Example 8 - LacZ Gene Transfer into Mouse

A. Transfer into Mouse Muscle

Five to six-week old male C57B/6 mice were anesthetized. Anterior tibialis muscles were exposed and directly injected with either rAd H5.000CBLacZ, H5.010CBLacZ or H5.001CBLacZ as follows: 25 μ L of purified viral suspension at a stock concentration of 5 \times 10¹¹ virus particles/mL was injected by inserting the tip of the 33 gauge needle of a 100 μ L Hamilton syringe into the belly of the muscle.

Animals were sacrificed on day 4, 14, 28 and 60 post injection. The muscles were dissected and frozen in liquid nitrogen cooled isopentane. Six μ M sections were cut in a cryostat, fixed and stained for β -galactosidase

activity for 6 hours at 37°C.

While the blue stained rAd was found for each virus in the day 4 and day 14 (most abundant) stains, by day 28, the H5.001CBLacZ clearly demonstrated more virus on day 28. By day 60, the only virus which stained positive was the H5.001CBLacZ.

5 B. *Transfer into Mouse Lung and Circulation*

RAd H5.000CBLacZ (control), and H5.001CBLacZ (1×10^{11} viral particles) were administered to six week old C57BL/6 female mice by tail vein injection and trachea installation. The animals were sacrificed and their liver and lung tissues were harvested at days 4, 9, 21, 28 and 35 post-administration. The transgene and viral late gene expression were compared.

10 15 At therapeutic doses of virus, there was diminished expression of late viral proteins at all time points in comparison with transgene.

C. *Dose Responses in Liver*

Dose responses of E4-deleted and E4 intact rAds in the liver of C57BL/6 mice were studied by tail vein administration of 1.5×10^{11} , 5×10^{10} , 1.7×10^{10} , 5.6×10^9 , and 1.9×10^9 viral particles and comparing the transgene and viral late gene expression at day 4, 21, 28, 35, and 42 post administration.

20 25 At therapeutic doses of virus, there was diminished expression of late viral proteins at all time points in comparison with transgene.

Example 9 - Other Gene Transfers

A. *Human OTC Gene Transfer*

30 The human OTC gene [A. L. Horwitz et al, *Science*, 224:1068-174 (1984)] or the human CFTR gene [Riordan et al, *Science*, 245:1066-1073 (1989)] was used to replace the LacZ as the transgene in the recombinant E1/E4 deleted adenoviruses described above, using the 35 techniques analogous for the construction of the above-

described *LacZ* vectors.

The resulting human OTC-containing rAd were administered at an MOI of 10 to 30 to human hepatocytes. The E1/E4 deleted rAd demonstrated less replication and 5 less late gene expression than when the E1/E4 deleted rAds are administered to muscle, as described in the example above. However, the results of this gene transfer are better than comparable transfers with rAds containing only a deletion in the E1 gene or a deletion 10 in the E1 gene and a point mutation in the E2a gene.

Similar results are demonstrated when the transgene is CFTR and the method of administration is intratracheal into lungs.

Example 10 - Transduction Efficiency of rAAV *LacZ* 15 AV.CMVLacZ) in HeLa Cells Infected with Ad Mutants

A. Viruses

The following viruses were employed in this experiment:

- (1) Wild-type Ad 5, propagated in 293 cells;
- 20 (2) Ad dl1110 (an Ad which is deleted of the 55 kb E1B gene) [Babiss et al, *J. Virol.*, 52(2):389-395 (1984) and Babiss and Ginsberg, *J. Virol.*, 50(1):202-212 (1984)], propagated in 293 cells,
- (3) H5.CBALP (an Ad deleted of its E1A and E1B 25 genes and containing a minigene that expresses alkaline phosphatase from a CMV enhanced β -actin promoter, as described above), propagated in 293 cells,
- (4) Ad ts125 (an Ad with a temperature sensitive mutation in the E2A gene which encodes the DNA 30 binding protein) [Ensinger and Ginsberg, *J. Virol.*, 10(3):328-339 (1972)], propagated in 293 cells,
- (5) Ad dl802 (an Ad deleted of its E2a gene), grown in E2A-complementing gmDBP cells as described in Rice and Klessig, *J. Virol.*, 56(3):767-778 (1985);

(6) Ad dl1004 (an Ad deleted of the E4 gene), grown in E4-complementing Vero W162 cells [Weinberg and Ketner, Proc. Natl. Acad. Sci. USA, 80(17):5383-5386 (1983)] and

5 (7) Ad dl1010 (an Ad deleted of ORF6 of its E4 gene), grown in E4-complementing Vero W162 cells [Weinberg and Ketner, cited above].

All viruses were purified by two sequential rounds of buoyant density ultracentrifugation in CsCl.

10 B. Experimental Procedures

HeLa cells seeded in 6 well, 36mm culture plates (2×10^6 cells/well) were infected with wild-type Ad5 or an adenovirus early gene mutant as described in Part A at an MOI of 10pfu/well. Infections were done in 15 1.0 ml DMEM/2%FBS. Six hours post-infection, monolayers were washed and 1.0 ml fresh DMEM/2% FBS media containing AV.CMVLacZ at 4×10^9 virus particles/ml were added.

Although the AV.CMVLacZ virus lot used in these experiments was shown to be free of H5.CLALP helper virus 20 by histochemical staining, the virus sample was subjected to heat treatment (60°C for 20 minutes) prior to use to ensure the absence of contaminating adenovirus. Two hours later, 1.0 ml of DMEM/115% FBS was added to each well.

25 Twenty-four hours after the addition of AV.CMVLacZ, cells were harvested. Each test condition was done in triplicate to enable virus transduction to be evaluated in terms of three outputs: histochemical staining for β -galactosidase activity (below), 30 intracellular β -galactosidase specific activity (Example 11), and the molecular form of the virus DNA (Example 12).

HeLa cells were histochemically stained for *E. coli* β -galactosidase (LacZ) activity as described in J. 35 M. Wilson et al, Proc. Natl. Acad. Sci. USA, 85:3014-3018

(1988). The different combinations that were tested included cells transfected with AAV vector alone (AV.CMVLacZ), vector plus wild-type Ad5 (+Ad5), vector plus dl110 (+dl110), vector plus Ad mutant H5.CBALP (+H5.CBALP), vector plus Ad mutant ts125 (+ts125), vector plus Ad mutant dl1802 (+dl1802), vector plus Ad mutant dl1004 (+dl1004), and vector plus Ad mutant dl1010 (+dl1010).

The results were observed in photomicrographs at magnification 10X (not pictured) of histochemical stains for recombinant β -galactosidase activity. The results indicated that wild-type Ad5 and the E2a mutants ts125 and dl1802 caused a significant increase in LacZ rAAV transduction as measured by the number of positive blue cells and the degree of stain intensity. Both dl110 (E1B-55kDa) and dl1010 (ORF6) enhanced transduction to levels that approached those of Ad5, ts125, and dl1802 in terms of the number of positive blue cells.

The E1 deletion recombinant H5.CBALP provided no significant increase in AV.CMVLacZ transduction. Expression of E1 alone was insufficient to have an effect in the adenovirus mediated augmentation of rAAV transduction as evidenced by lack of significant increase in transduction obtained with HeLa cells infected with the E4 deletion mutant dl1004. A significant drop in transduction occurred following removal of ORF6 from the E4 region from the coinfecting adenovirus (Fig. 5A).

It is believed that these results demonstrate that the adenoviral gene products, E4 and E1 indirectly promote the formation of ds DNA intermediates that are transcriptionally active.

Example 11 - Quantitation of Enhanced Vector Transduction

(A) A duplicate set of HeLa cells as described in Example 10B were used in this experiment. Twenty-four hours after the addition of AV.CMVLacZ recombinant, for

intracellular β -galactosidase assays, cell pellets were suspended in 0.5 ml PBS and sonicated. Cell debris was removed by centrifugation (15,000Xg for 10 minutes) and the clarified extract assayed for total protein [M.

5 Bradford, Anal. Biochem., 72(1-2):248-254 (1976) and M. Bradford et al, Fed. Proc., 35(3):274 (1976)] and β -galactosidase activity [Sambrook et al, cited above] using *o*-nitrophenyl β -D-galactopyranoside (ONPG) as substrate.

10 Fig. 5A demonstrates the transduction efficiency quantitated by measuring β -galactosidase enzyme activity in the lysates from infected HeLa cells and also assayed for total protein. In Fig. 5A, the test condition is shown along the horizontal axis, and
15 intracellular β -galactosidase specific activity (milliunits/mg protein) using ONPG as substrate is plotted on the vertical axis. Below each bar, the fold-induction in specific activity relative to cells that received the AV.CMVLacZ vector alone is given.

20 The results of Fig. 5A demonstrate that the E2a mutants ts125 and dl802 produced 134-fold and 225-fold increases in β -galactosidase activity, respectively, as compared to that achieved with purified rAAV alone. In comparison, cells infected with wt Ad5 generated 107-fold
25 increase in β -galactosidase activity.

(B) In another experiment, HeLa cells (2×10^6) were infected with increasing multiplicities of wild-type Ad5 or the E2 mutant dl802. Six hours post-infection, monolayers were washed and infected with AV.CMVLacZ at
30 1000 virus particles/cell. Twenty-four hours after the addition of AV.CMVLacZ, cells were harvested and assayed for total protein and β -galactosidase activity.

The results are illustrated in the bar graph of Fig. 5B, in which adenovirus MOI's are given along the
35 horizontal axis, and intracellular β -galactosidase

specific activity along the vertical axis. Enhancement of rAAV transduction was proportional to input helper adenovirus from MOIs of 1 to 50 for both wild type Ad5 and dl802. Higher doses of virus were cytopathic,

5 leading to a fall in β -galactosidase expression.

Enhanced transduction was achieved when the cells were infected prior to, or at the time of, rAAV infection.

The E1 deletion recombinant H5.CBALP and the E4 deletion mutant dl1004 provided no significant increase in

10 AV.CMVLacZ transduction. Both cells infected with dl110 (E1B-55kDa) and with dl1010 (ORF6) demonstrated substantially lower total β -galactosidase activity than those infected with Ad5, ts125, or dl802.

Example 12 - Analysis of Low Molecular Weight DNAs in

15 AV.CMVLacZ Transduced Cells

Studies with these early gene mutants of adenovirus suggested that expression of adenoviral genes rather than the virion itself was responsible for enhancement of rAAV transduction. To further investigate these mechanisms

20 and to determine if conversion of ss to ds genome limits the transduction efficiency of rAAV, the molecular state of the rAAV genome was characterized in the infected cells. The relationship between RFm formation and lacZ rAAV transduction was explored in experiments where the 25 dose of coinfecting virus was varied (MOI=1, 5, or 10).

(A) A duplicate set of HeLa monolayers as described in Example 10 were harvested 24 hours after they were transduced with the recombinant AV.CMVLacZ and cultured with or without helper adenovirus.

30 Episomal DNA was extracted from cell pellets using a modification of the procedure originally described by B. Hirt, J. Mol. Biol., 26:365-369 (1967). Briefly, cells were suspended in 320 ml Tris-Cl (pH8.0)/10 mM EDTA and SDS added to a final concentration 35 of 1%. The mixture was incubated at 37°C for 30 minutes.

Pronase and proteinase K were added to final concentrations of 500 μ g/ml and 20 μ g/ml, respectively, and the reaction incubated at 37°C for 2 hours. Sodium chloride was added to a final concentration of 1.1 M and 5 incubated at 4°C overnight. The precipitate that developed during the 4°C incubation was pelleted at 20,000 xg for 30 minutes and the clear supernatant carefully removed. The supernatant was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) followed 10 by chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with ethanol. The final pellet was suspended in 50 μ l Tris-Cl (pH 8.0)/1.0 mM EDTA.

These Hirt extracts were analyzed by Southern blot hybridization. Samples (5 μ l) of each Hirt extract 15 were resolved through a 1.2% agarose gel, electroblotted onto a nylon membrane and hybridized with a 32 P random primer labeled cDNA of the SV40 polyA signal used in AV.CMVLacZ.

An autoradiogram of the experiment of Example 20 12 (not pictured), identifies and labels bands corresponding to the ss AV.CMVLacZ genome (SS), a monomer replicative form (RFm), and concatomer replicative forms (RFd). Bands corresponding to the ss AV.CMVLacZ genome (SS), a monomer replicative form (RFm), and concatomer 25 replicative forms (RFd) were identified and labeled. To reference the RFm band, a plasmid carrying AV.CMVLacZ was digested to release the entire genome. Autoradiogram exposure times were 14 hours and 69 hours.

In this autoradiogram, the full spectrum of 30 molecular species present during a lytic infection was demonstrated in cells infected with both LacZ rAAV and wild type adenovirus. Both the input ss genome (SS) and monomeric and dimeric forms of ds replicative intermediates (RFm and RFd) are present. This contrasts 35 with cells infected with purified rAAV alone, where ss

5 genome is the sole molecular form detected. Analysis of cells coinfecte^d with the adenovirus early gene mutants revealed a direct correlation between formation of ds forms of the rAAV genome and the enhancement of *LacZ* transduction. Mutant adenoviruses that were ineffective in enhancing rAAV transduction (i.e., the E1 deleted mutant H5.CBALP and the E4 deleted mutant dl1004) failed to promote the formation of ds forms of AAV.

10 Cells infected with adenovirus deleted of E2a (dl802) or partially deleted of E1 (dl110) or E4 (dl1010) additionally demonstrated a band whose size was identical to the ds replicative monomer (RFm) of the *lacZ* rAAV genome and whose abundance correlated directly with the expression of β -galactosidase activity (compare results 15 of Example 14 to these described results). Slower migrating concatomers, likely dimers, of duplex rAAV were also detected in the autoradiogram described above.

20 In the presence of E1 and E4 expression, rAd transduction was invariably accompanied by the appearance of ds RF monomers and dimers.

The high molecular weight band in sample lane +H5.CBALP is helper virus DNA. Helper virus DNA is recognized by the SV40 probe because the CBALP minigene also utilizes the SV40 polyA signal.

25 (B) In another experiment, HeLa cells were infected with wt Ad5 or the E2 deleted mutant dl802 as described in Example 10B. Monolayers were harvested 24 hours later and analyzed for β -galactosidase activity and RFm synthesis. Monomer bands similar to those shown in the 30 autoradiogram described above were quantitated on a PhosphorImager 445 SI and assigned values (CPM).

The results are illustrated in the graphs of Figs. 8A and 8B, in which β -galactosidase specific activity and CPM are plotted along the vertical axis of 35 each figure. Adenovirus MOI's are given on the horizontal

axis of each figure. Data obtained from low MOI infections (1, 5, and 10) are shown. Importantly, the tight correlation between rAAV vector transduction and the accumulation of duplex forms could be achieved in 5 cells infected with E1/E4 expressing adenovirus. The level of β -galactosidase and abundance of RFm increased in proportion to the amount of infecting wild type Ad (Fig. 6A) and d1802 (Fig 6B). These data suggest that synthesis of an episomal duplex intermediate is an 10 obligatory event in transduction.

Example 13 - Duplex End-Analysis

The following is a description of a model for leading strand synthesis of a complementary AAV strand in the presence of Rep (+Rep) or absence of Rep (-Rep). 15 Refer to Figs. 7A-7F. Rep expresses a terminal resolution activity that can convert a duplex structure with closed-ends to an open-ended duplex. In the absence of Rep, terminal resolution is impaired leaving the covalently closed, hairpin structures intact. Under 20 these conditions, hairpins are expected to be found leftward and rightward, since both strands of a rescued ds AAV genome are packaged into virions. Figs. 7B-7F are a flow chart demonstrating the strategy for identifying the terminal structure of duplex RFm that is synthesized 25 from ss AV.CMVLacZ in response to adenoviral gene expression.

Fig. 7C illustrates a closed end and an open end fragment of rAV.CMVLacZ. Figs. 7D, 7E and 7F indicate the mixture of open-ended and covalently closed duplex 30 fragments generated by NotI digestion at position 4509 in the absence of terminal resolution. The NotI 4509 digestion provides a convenient means of releasing a 361 bp fragment that contains the right ITR in the context of a hybridization target (i.e. SV40 pA). In the presence 35 of terminal resolution, only the open-ended 361 bp

fragment would be expected to be generated (Fig. 7D) by such digestion.

The resulting electrophoretic gel (not pictured), revealed in lane (1) the results of digestion of a 5 plasmid carrying an AV.CMVLacZ cDNA to release the rAAV vector and subsequent digestion with NotI to release the right terminal 361 bp fragment. In lane (2) a sample of NotI digested Hirt DNA extracted from HeLa cells infected with wild-type Ad5 and transduced with AV.CMVLacZ 10 resulted in the release of two fragments, labeled FormI and FormII. (See, also, Figs. 8A and 8B). The migration of ss AV.CMVLacZ (SS) and RFm were also seen.

The ds AV.CMVLacZ intermediates that accumulated in cells infected with adenovirus were likely the result of 15 leading strand DNA synthesis, initiating from the duplex region of the vector ITR. In the absence of Rep, this conversion event was anticipated to generate molecules in which one end is open and the other is covalently closed (Fig. 7A). To further characterize the structure of this 20 ds intermediate Hirt extracts from cells coinfecte with rAV.CMVLacZ and Ad5 were digested with NotI to release the termini of the ds intermediate which, if left open, would be approximately 361 bp in length. The resulting filters were hybridized with a probe specific for the 25 SV40 polyadenylation signal positioned immediately upstream of the rightward ITR. At least two forms were released from the right end of duplex genomes, one that migrated to a position in the gel that predicted an open-ended conformation (Form II), and a second slower 30 migrating species (Form I). Although this result was consistent with the model (Figs. 7A-7F), it was difficult to predict with certainty the structure of Form I. Its retarded mobility did, however, suggest a conformation that differed from the open-ended Form II.

Example 14 - Analysis of AV.CMVLacZ TransductionEfficiency in 293 Cells Stably Transfected with an
Inducible E4 ORF6 cDNA

Cell lines used in this assay were prepared as
5 described in Example 1. 293(MT-ORF6) cells and HeLa(MT-
ORF6) cells were seeded in 6 well 35 mm culture plates
(2×10^6 cells/well) and infected with purified, heat-
treated AV.CMVLacZ at an MOI of 1000 virus
particles/cell. Induction of ORF6 expression with from
10 none to increasing concentrations of zinc sulfate was
initiated 2 hours before the addition of virus and
continued throughout the duration of the experiment.

Twenty-four hours after the addition of virus, cells
were harvested, lysates were generated by sonication and
15 analyzed for the β -galactosidase expression (i.e.,
 β -galactosidase activity) and virus DNA as described in
the preceding examples. Hirt extracts were prepared from
low molecular weight DNA from cell extracts. The
preparation of the Hirt extracts and subsequent analysis
20 by Southern hybridization were performed similarly to
those described in the examples above.

The results of this experiment were as follows:

(1) Specific Activity

The results are illustrated in the bar
25 graph of Fig. 8A. Specific activity (milliunits
 β -galactosidase/mg protein) is plotted along the vertical
axis. Below each bar is given the concentration of zinc
used for induction, the fold-induction relative to 293
cells, and the fold-induction relative to 293(ORF6) cells
30 maintained in the absence of zinc. As shown in Fig. 8A,
in the absence of Zn^{+2} , the 293(MT-ORF6) cell line
generated 39-fold higher levels of β -galactosidase in
rAAV infected 293 cells. Induction of ORF6 expression
with increasing amounts of Zn^{+2} resulted in a concomitant
35 rise in AV.CMVLacZ cell transduction to a level that was

445-fold greater than the parent 293 line. Expression of E1 alone was insufficient to have an effect in the adenovirus mediated augmentation of rAAV transduction.

The specific activity of β -galactosidase was 196.2 mUnits/mg in E1/E4 expressing 293 cells, compared to 1.0 mUnit/mg in 293 cells that only expressed E1 genes. These experiments support a mechanism for enhancing rAAV transduction that is dependent on the combined expression of both E1 and E4 adenoviral genes.

10 (2) Molecular Analysis of the AV.CMVLacZ

Genome

The duplex monomer replicative form (RFm) was quantitated and the values (CPM) plotted along the vertical axis in the bar graph of Fig. 8B. The 15 concentration of zinc used for induction and the fold-induction relative to 293(ORF6) cells maintained in 0 mM zinc is given below each bar.

An autoradiogram (not pictured) shows the agarose gel resolved Hirt extracts from the AV.CMVLacZ 20 transduced cells described above. A plasmid carrying the AV.CMVLacZ cDNA was digested to release the entire sequence and loaded in a lane of the autoradiogram. The band that appeared in this lane therefore reflected the migration of a monomer duplex replicative form (RFm). 25 The migration of the ss AV.CMVLacZ genome (SS), RFm, and dimers of the duplex replicative form (RFd) were also shown. Lanes of the autoradiogram labeled (0), (50), (100), (150), (200), and (250) contained samples from 293(MT-ORF6) cells that were induced with the indicated 30 concentration of zinc. A Hirt extract from 293 cells (lane labeled 293) transduced with AV.CMVLacZ was also shown.

35 Analysis of Hirt extracts revealed the presence of the RFm in the rAAV infected 293(MT-ORF6) cells that was not present in similarly infected 293

cells. When the induction profiles (Figs. 8A and 8B) that describe AV.CMVLacZ transduction efficiency were compared, the results were plotted in Fig. 8C. Specific activity (milliunits β -galactosidase/mg protein) data from Fig. 8A and counts-per-minute data (CPM) of AV.CMVLacZ RFm from Fig. 8B are plotted along the vertical axis, and concentration of zinc sulfate used during the experiment is shown along the horizontal axis.

The two profiles are near mirror images. Importantly, the RFm increased in proportion to the increment in lacZ transducing activity that occurred as ORF-6 expression was induced with Zn^{+2} (Fig. 8C). Similar results were obtained with a 293 derived cell line that expresses ORF6 from the glucocorticoid responsive MMTV promoter.

Example 15 - Enhanced AV.CMVLacZ Transduction in HeLa Cells Carrying an Inducible ORF6 Minigene

HeLa(MT-ORF6) cells (2×10^6) were transduced at an MOI of 1,000 AV.CMVLacZ recombinant particles/cell in absence of zinc sulfate inducer or in the presence of 50, 100, 150, 200, or 250 μ M zinc sulfate inducer in the media during transduction. Twenty-four hours later, cells were harvested, cell extracts were prepared by sonication, and analyzed for transgene expression (i.e., β -galactosidase activity). Cell monolayers were histochemically stained for β -galactosidase activity.

The resulting photomicrographs (not pictured) illustrated that histochemical staining revealed an increase in the number of cells scored lacZ positive as the concentration of Zn^{+2} in the medium was raised from 0 to 200 mM. Concentrations of 250 mM zinc were found to be toxic to the cells.

Specific activity (milliunits β -galactosidase/mg protein) is plotted in Fig. 9 along the vertical axis. Below each bar is given the concentration of zinc used

for induction, the fold-induction relative to HeLa cells, and the fold-induction relative to HeLa(Mt-ORF6) cells maintained in the absence of zinc. Histochemical staining revealed an increase in the amount of

5 β -galactosidase in lysates as the concentration of Zn^{+2} in the medium was raised from 0 to 200 mM.

Example 16 - Southern Blot Analysis of Low Molecular Weight DNAs from AV.CMVLacZ Transduced HeLa(MT-ORF6) Cells

10 Following Induction of E4ORF6

Hirt extracts were prepared from HeLa(MT-ORF6) cells transduced with AV.CMVLacZ as described in Example 15 in the presence of increasing concentrations of Zn^{+2} to determine whether synthesis of duplex intermediates 15 contributed to the augmentation in AV.CMVLacZ transduction.

20 Samples of HeLa(MT-ORF6) cells that were induced with a concentration of zinc sulfate (0, 50, 100, 150, 200, and 250) were resolved on a 1.2% agarose Southern gel (not pictured), transferred to a nylon membrane, and hybridized with a LacZ-specific probe. One lane contained a plasmid encoding AV.CMVLacZ that was digested to release the entire genome. Bands corresponding to the ss AV.CMVLacZ genome (SS), duplex monomers (RFm), and 25 duplex dimers (RFd) were indicated on the gel.

25 Southern analysis indicated that Hela and uninduced Hela(MT-ORF6) cells demonstrated a single band on Southern blots which comigrated with the ss genome. Induction of ORF-6 resulted in the appearance of 30 detectable levels of ds monomer but only at higher concentrations of Zn^{+2} . A band comigrating with the RFd was present in all cell preparations, the relevance of which is unclear since the monomer is a likely precursor to the dimer.

Example 17 - Effect of Adenovirus Infection on In Vivo AV.CMVLacZ Targeting Efficiency To Murine Liver

5 The impact of adenoviral gene expression on rAAV transduction in murine liver was studied by sequentially infusing into the portal vein early gene mutants of adenovirus followed by rAAV.

10 Balb/c mice, 4- to 6-weeks old [Jackson Laboratories, Bar Harbor, Maine] were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (10 mg/kg). For liver studies, a 1 cm left flank incision was made and the spleen exposed.

15 Samples of purified, heat-treated AV.CMVLacZ in 50 μ l HBS (1×10^{11} virus particles) were used alone or spiked with helper adenovirus containing 2×10^{10} A₂₆₀ particles of purified dl1004, H5.CBALP, or ts125 in a final volume of 50 μ l. The dose of adenovirus was sufficient to transduce >25% of hepatocytes. The virus mixture was injected just beneath the splenic capsule and the abdomen was closed with 3-0 vicryl.

20 Necropsies were performed 3 days post-infusion and tissue frozen in O.C.T. embedding compound. Frozen sections (6 μ m) (LacZ+ALP) were prepared and histochemically stained for β -galactosidase enzyme and alkaline phosphatase activity. Sections were 25 counterstained with neutral red and mounted.

A β -galactosidase positive hepatocyte targeted with AV.CMVLacZ at magnification 20X was obtained. Histochemical analyses of liver tissue harvested 3 days after gene transfer demonstrated that administration of 30 10^{11} particles of purified rAV.CMVLacZ alone into the portal vein was not associated with appreciable gene transfer (<0.01% of cells), confirming the inherent inefficiency of the rAAV system.

35 Preinfusion with E4 deleted virus had no impact on rAAV transduction in mouse liver, whereas E1 deleted

virus demonstrated a modest increment in lacZ positive hepatocytes to about 0.1%. The most significant increase in rAAV transduction occurred following infusion of the E2a adenovirus mutant ts125 with lacZ expression detected 5 in 10-25% of hepatocytes. A direct relationship between adenovirus gene expression and rAAV transduction was demonstrated in animals infused with both lacZ rAAV and the ALP expressing E1 deleted virus. The dose of adenovirus was reduced 10-fold to minimize the 10 coincidental occurrence of coinfection. Histochemical studies demonstrated co-localization of ALP and β -galactosidase in the majority of β -galactosidase expressing hepatocytes.

Example 18 - Effect of Adenovirus Infection on In Vivo 15 AV.CMV LacZ Targeting Efficiency To Murine Lung

Experiments described in Example 17 for mouse liver were adapted for the study of rAAV mediated gene transfer to mouse lung. For lung experiments, anesthetized Balb/C animals were intubated as described in DeMatteo et al, 20 Transplantation (Baltimore), 59(5):787-789 (1995). Briefly, a midline 2 cm skin incision was made in the neck to expose the trachea. A 2 inch 18 gauge angiocatheter was passed through the mouth, positioned in the midportion of the trachea, and connected to a rodent 25 ventilator (#55-3438 Harvard). Polyethylene (PE#10, Intramedic) was fed through the catheter via a side port and advanced beyond the tracheal bifurcation. Using a Hamilton syringe, virus samples (30 μ l) were slowly infused into the lung through the polyethylene tubing. 30 Samples contained the same formulation of purified, heat-treated AV.CMV LacZ with or without helper adenovirus, as described for liver injections.

Tissue was harvested 72 hours post-infusion. Frozen sections were histochemically stained for β -galactosidase 35 activity and counterstained with neutral red.

Frozen sections from lung (AV.CMVLacZ) showed a β -galactosidase positive airway epithelial cell targeted with AV.CMVLacZ. Similar studies were performed in the murine model of lung-directed gene transfer.

5 Adenoviruses were instilled into the trachea prior to the instillation of rAAV. Analysis of lung tissue 3 days later revealed only a rare β -galactosidase positive cell in animals instilled with rAAV alone. No detectable enhancement of rAAV transduction was noted in animals
10 preinstilled with adenovirus deleted of either E1 or E4. Substantial enhancement of transduction was achieved in conducting airway and alveolar cells of animals administered the E2a mutant adenovirus.

These experiments in murine models of gene therapy
15 directed to liver and lung verified that the efficiency of rAAV transduction is low due limited conversion of the input ss genome to a transcriptionally active ds intermediate, and that this conversion is facilitated by expression of adenovirus E1 and E4 gene products.

20 Example 19 - Second Generation rAAV with Regulated Minigene Capable of Enhancing Transduction

The experiments described in previous examples illustrated the following principles: 1) purified rAAV is a relatively inefficient gene transfer vehicle *in vitro*
25 and *in vivo* and 2) the rate limiting step in transduction is not viral entry but rather conversion of the virion's ss DNA genome to a transcriptionally active ds DNA genome. Adenovirus can substantially enhance transduction through expression of a subset of its genes.
30 It does this by promoting conversion of the virion's genome to its ds form. One approach to accomplish this is to incorporate into the recombinant AAV genome a minigene that expresses the minimal adenoviral genes necessary to enhance transduction, i.e., the ORF6 region
35 of E4.

Two approaches have been considered in designing this modified rAAV. The first strategy is based on a rAAV genome that has two transcriptional units in series, one expressing the therapeutic gene and the other 5 expressing its E4 ORF6 from a constitutive promoter. While this may, in fact, be useful in many situations, constitutive expression of ORF6 may be detrimental to the cell and potentially could elicit a destructive immune response.

10 The second version of this rAAV includes the therapeutic minigene in addition to the ORF6 transcriptional unit which, in this case, is expressed from an inducible promoter. When this second gene rAAV is administered to the cells (ex vivo strategies) or to 15 the patient (in vivo strategies), the inducing agents are administered at the time of gene transfer or soon thereafter. If the ds genomic form or its integrated derivative is stable, the induction of ORF6 will only be necessary at the time of gene transfer into the recipient 20 cell. Following this, its inducing agent will be withdrawn and the ORF6 gene will be turned off.

An rAAV that illustrates this concept of inducible ORF6 has been constructed and tested *in vitro*. A schematic of the vector pAV.CMVALP.GRE-ORF6, is shown in 25 Fig. 11 and its sequence is illustrated in SEQ ID NO: 5. This second generation construct contains flanking 5' and 3' AAV ITR sequences. The human placental alkaline phosphatase cDNA (ALP) is included in a minigene in which the promoter from the immediate early gene of 30 cytomegalovirus drives the transcription. A second transcriptional unit is cloned between the ITRs in series and in direct orientation with the alkaline phosphatase minigene. The second transcriptional unit expresses the Ad5-E4-ORF6 from a glucocorticoid dependent promoter 35 (GRE) with an SV40 polyadenylation signal. This is

called a second generation rAAV construct.

Specifically, pAV.CMVALP.GRE-ORF6 [SEQ ID NO: 5] generates a novel rAAV containing the *LacZ* transgene and the Ad E4 ORF 6 which facilitates ss to ds conversion of 5 rAAV. The plasmid includes a flanking AAV 5' ITR sequence (nucs. 53-219); CMV enhancer/promoter (nucs. 255-848); human placenta alkaline phosphatase cDNA (ALP) (nucs. 914-2892); SV40 polyA (nucs. 2893-3090); GRE promoter (nucs. 3114-3393); Ad5 E4-ORF6 cDNA (nucs. 3402-10 4286); SV40 polyA (nucs. 4315-4512); and 3' AAV ITR (nucs. 4547-4713). All other nucleotides are plasmid derived.

The second generation rAAV construct was used to produce and purify rAAV virions which were exposed to 15 HeLa cells that were left untreated or incubated with dexamethasone. In the absence of dexamethasone, (a condition under which little ORF6 should be expressed), little transduction was observed as measured by expression of the alkaline phosphatase gene. Cells 20 incubated in dexamethasone expressed in ORF6 gene and the transduction efficacy was enhanced at least 5-fold. This provides evidence to support that a gene product expressed from the rAAV can function in *cis* to enhance expression of the transgene.

25 Example 20 - Application to Bone Marrow Directed Gene Therapy

Bone marrow directed gene therapy represents the paradigm of *ex vivo* gene therapy where the target cell is the hematopoietic stem cell. The basic strategy is to 30 incorporate (i.e., integrate) a therapeutic minigene into the chromosomal DNA of hematopoietic stem cells which are transplanted into a recipient patient whose own bone marrow has been ablated allowing repopulation of its lymphohematopoietic system with progeny of the 35 genetically corrected stem cell.

The problem with this approach has been efficiently transfecting genes into stem cells. Most studies of bone marrow directed gene therapy have utilized recombinant retroviruses which have not been very efficient. One 5 problem is that retroviruses integrate their provirus only when the target cell is dividing. Unfortunately, most stem cells *in vitro* are quiescent and not dividing. rAAV holds the promise of integrating the provirus more efficiently into non-dividing stem cells. However, 10 purified rAAV is not very efficient with respect to integration when used alone. In cultured cells, integration is observed in less than 1% of the cells. The same conditions that activate the conversion of ss to ds genome also enhance the integration of the ds 15 intermediate into the chromosomal DNA.

Therefore, a desirable application of the methods and compositions of this invention is in bone marrow directed gene therapy. According to this method, stem cells are genetically modified with rAAV and an inducing 20 agent *ex vivo* using the constructs and methods described above (see e.g., Example 19). Genetically modified stem cells are subsequently transplanted by conventional techniques.

Numerous modifications and variations of the present 25 invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention, such as selections of different transgenes and plasmids 30 for the construction of the packaging cell lines and rAds, or selection or dosage of the viruses or immune modulators, are believed to be encompassed in the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Trustees of the University of Pennsylvania
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(ii) TITLE OF INVENTION: Recombinant Adenovirus and Adeno-
Associated Virus, Cell Lines,
and Methods of Production
and Use Thereof

(iii) NUMBER OF SEQUENCES: 5

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release 1.0 Version 1.30

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(B) FILING DATE:
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(B) FILING DATE: 05-JUN-1995

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3653 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1521..2405

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCATGTGT CAGAGGTTT CACCGTCATC ACCGAAACGC GCGAGGCAGC	50
AAGCTTGGCA GAAATGGTTG AACTCCCGAG AGTGTCTTAC ACCTAGGGGA	100
GAAGCAGCCA AGGGGTTGTT TCCCACCAAG GACGACCCGT CTGCGCACAA	150
ACGGATGAGC CCATCAGACA AAGACATATT CATTCTCTGC TGCAAACATTG	200
GCATAGCTCT GCTTGCCTG GGGCTATTGG GGGAAAGTTGC GGTCGTGCT	250
CCGAGGGCTC TCACCCCTGA CTCTTCAAT AATAACTCTT CTGTGCAAGA	300
TTACAATCTA AACAAATTCGG AGAAACTCGAC CTTCCTCCTG AGGCAAGGAC	350
CACAGCCAAC TTCCTCTTAC AAGCCGCATC GATTTGTCC TTCAGAAATA	400
GAAATAAGAA TGCTTGCTAA AAATTATATT TTTACCAATA AGACCAATCC	450
AATAGGTAGA TTATTAGTTA CTATGTTAAG AAATGAATCA TTATCTTTA	500
GTACTATTT TACTCAAATT CAGAAGTTAG AAATGGGAAT AGAAAATAGA	550
AAGAGACGCT CAACCTCAAT TGAAGAACAG GTGCAAGGAC TATTGACCAC	600
AGGCCTAGAA GTAAAAAAGG GAAAAAAGAG TGTTTTGTC AAAATAGGAG	650
ACAGGTGGTG GCAACCAGGG ACTTATAGGG GACCTTACAT CTACAGACCA	700
ACAGATGCC CTTTACCAT A TACAGGAAGA TATGACTTAA ATTGGGATAG	750
GTGGGTTACA GTCAATGGCT ATAAAGTGT T ATATAGATCC CTCCCCTTTC	800
GTGAAAGACT CGCCAGAGCT AGACCTCCTT GGTGTATGTT GTCTCAAGAA	850
AAGAAAGACG ACATGAAACA ACAGGTACAT GATTATATT ATCTAGGAAC	900

AGGAATGCAC	TTTTGGGAA	AGATTTCCA	TACCAAGGAG	GGGACAGTGG	950							
CTGGACTAAT	AGAACATTAT	TCTGCAAAA	CTTATGGCAT	GAGTTATTAT	1000							
GATTAGCCTT	GATTTGCCCA	ACCTTGCAGT	TCCCAAGGCT	TAAGTAAGTT	1050							
TTTGGTTACA	AACTGTTCTT	AAAACAAGGA	TGTGAGACAA	GTGGTTTCCT	1100							
GA	CTTGGTTT	GGTATCAAAG	GTTCTGATCT	GAGCTCTGAG	TGTTCTATT	1150						
TCCTATGTT	TTTTGGAATT	TATCCAAATC	TTATGTAAAT	GCTTATGTAA	1200							
ACCAAGATAT	AAAAGAGTGC	TGATTTTTG	AGTAAACTG	CAACAGTCCT	1250							
AA	CATTCA	TCTTGTGTGT	TTGTGTCTGT	TCGCCATCCC	GTCTCCGCTC	1300						
GTCAC	TTACTTATC	CTTCAC	CAGAGGGTCC	CCCCGCAGAC	CCCGGCGACC	1350						
CTCAGGTCGG	CCGACTGCGG	CAGCTGGCGC	CCGAACAGGG	ACCCCTCGGAT	1400							
AA	AGTAC	CCCT	TGTCTTTATT	TCTACTATT	TGTGTTCGTC	TTGTTTGTC	1450					
TCTATCTG	TG	GGCTATCA	TCACAAGAGC	GGAACGGACT	CACCTCAGGG	1500						
AA	CCAAG	CTA	GCCCAATT	CG	ATGACTACGT	CCGGCGTTCC	ATTGGCATG	1550				
AC	ACTAC	CGAC	AAACACGATC	TCGGTTGTCT	CGGCGCACTC	CGTACAGTAG	1600					
GG	ATCGT	CTA	CCTCCTTTG	AGACAGAAAC	CCGGCGCTACC	ATACTGGAGG	1650					
ATC	ATCCG	GCT	GCCC	GAA	TGTAACACTT	TGACAATGCA	CAACGTGAGT	1700				
TAC	GTGCG	GAG	GT	CTTCC	CTG	CAGTGTGG	TTTACGCTGA	TTCAGGAATG	1750			
GG	TTGTT	CCC	TGG	GATAT	GG	TTCTAACGCG	GGAGGGAGCTT	GTAATCCTGA	1800			
GGA	AGTGT	TAT	GCACGTGT	GC	CTGTGT	GG	CCAACATT	GA	TATCATGACG	1850		
AGC	ATGAT	GA	TCC	CATGG	TA	CGAGTC	CTGG	GC	ACT	GTCATTGTT	1900	
CAG	TCC	CGG	T	CC	GT	GT	AGC	GG	CAGG	TTGGCCAGCT	1950	
GG	TTT	AGG	GAT	GG	GT	GG	GG	CAT	GT	TTATATGG	2000	
TAC	CGG	GAG	G	GG	GT	GA	AA	AGAG	GT	TTATATGG	2050	
CAG	CGT	GT	TT	ATGAGGG	GC	CA	ACAT	GCC	AT	ACAGCGCC	2100	
ATG	GGCC	ACGT	GG	GGT	CTG	TG	CCCC	GCCA	TG	AGCTTTGG	2150	
TTG	CA	GTG	GG	ATTT	GAA	CA	ATATT	GTG	GTG	GCT	GCAGTTACTG	2200

TGCTGATTTA	AGTGAGATCA	GGGTGCGCTG	CTGTGCCCGG	AGGACAAGGC	2250
GCCTTATGCT	GCGGGCGGTG	CGAATCATCG	CTGAGGAGAC	CACTGCCATG	2300
TTGTATTCT	GCAGGACGGA	GCGGCGGCCG	CAGCAGTTA	TTCGCGCGCT	2350
GCTGCAGCAC	CACCGCCCTA	TCCTGATGCA	CGATTATGAC	TCTACCCCCA	2400
TGTAGGGATC	CAAGCTTGC	GGCGCATCGA	TGATATCAAG	CTTGCATGCC	2450
TGCAGGTCGA	CTCTAGAGGA	TCCCGGGTGG	NATCCCTGTG	ACCCCTCCCC	2500
AGTGCCTCTC	CTGGCCCTGG	AAGTTGGCAC	TCCAGTGCC	ACCAGCCTTG	2550
TCCTAATAAA	ATTAAGTTGN	ATCATTGT	CTGACTAGGT	GTCCTTCTAT	2600
AATATTATGG	GGTGGAGGGG	GGTGGTATGG	AGCAANGGN	AANTTGGNAA	2650
GACAANCTGT	AGGGCCTGCG	GGGTCTATTG	GGAACAAGCT	GGAGTGCAGT	2700
GGCACAACTCT	TGGCTCACTG	CAATCTCCGC	CTCCTGGGTT	CAAGCGATT	2750
TCCTGCCTCA	GACTCCCGAG	TTGTTGGGAT	TCCAGGCATG	CATGACCAGG	2800
CTCAGATAAT	TTTGTTTTT	TTGGTAGAGA	CGGGGTTCA	CCATATTGGN	2850
CAGGCTGGTC	TCCAACTCCT	AATCTCAGGT	GATCTNCCCA	CCTTGGCCTC	2900
CCAAATTGCT	GGGATTACAG	GNGTGAACCA	CTGNTCCCTT	CCCTGTCCTT	2950
CTGATTTAA	AATAACTATA	CCAGCAGGAG	GACGTCCAGA	CACAGCATAG	3000
GCTACCTGGC	CATGCCAAC	CGGTGGGACA	TTTGAGTTGC	TTGCTTGGCA	3050
CTGTCCTCTC	ATGCGTTGGG	TCCACTCAGT	AGATGCCTGT	TGAATTGGGT	3100
ACGCGGCCAG	CTTGGCTGTG	GAATGTGTGT	CAGTTAGGGT	GTGGAAAGTC	3150
CCCAGGCTCC	CCAGCAGGCA	GAAGTATGCA	AAGCATGCAT	CTCAATTAGT	3200
CAGCAACCAG	GTGTGGAAAG	TCCCCAGGCT	CCCCAGCAGG	CAGAAAGTATG	3250
CAAAGCATGC	ATCTCAATTA	GTCAGNAACC	ATAGNCCCGC	CCCTAACTCC	3300
GTCCATCCCG	GCCCTAACTC	NGGCCAGTTC	CGACCNTNCT	CCGGCNNATG	3350
GNTGAGTAAT	TTGCNNGATT	TATGCAGNGG	GCGAGGNCGC	CTCGGGCTCT	3400
GAGNTNTTCC	AGAAGTAGTG	AGGAGGCTTT	NNTGGTGGAA	TTGATCAGCT	3450
TGGGATCTGA	TCAAGAGACA	GGATGAGGAT	CGNNNCGNAT	GATTGAACAA	3500

72

GATGGGTTGC ACGGAGGTTTC TCCGGNCGCT TGGGTGGGGA GGNTATTCGG	3550
NTATTNTTGG TGNACAAACAG NNAAACGGNT GTTCTGATGC CGCCGCGTTC	3600
NCGCTTTCAG NGCAGGGGGG CCCCCCTTCT NTTGAGANNA GCNCCCCTTN	3650
TTG	3653

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 294 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Thr Thr Ser Gly Val Pro Phe Gly Met Thr Leu Arg Pro Thr Arg	
1 5 10 15	
Ser Arg Leu Ser Arg Arg Thr Pro Tyr Ser Arg Asp Arg Leu Pro Pro	
20 25 30	
Phe Glu Thr Glu Thr Arg Ala Thr Ile Leu Glu Asp His Pro Leu Leu	
35 40 45	
Pro Glu Cys Asn Thr Leu Thr Met His Asn Val Ser Tyr Val Arg Gly	
50 55 60	
Leu Pro Cys Ser Val Gly Phe Thr Leu Ile Gln Glu Trp Val Val Pro	
65 70 75 80	
Trp Asp Met Val Leu Thr Arg Glu Glu Leu Val Ile Leu Arg Lys Cys	
85 90 95	
Met His Val Cys Leu Cys Cys Ala Asn Ile Asp Ile Met Thr Ser Met	
100 105 110	
Met Ile Tyr Gly Tyr Glu Ser Trp Ala Leu His Cys His Cys Ser Ser	
115 120 125	
Pro Gly Ser Leu Gln Cys Ile Ala Gly Gly Gln Val Leu Ala Ser Trp	
130 135 140	
Phe Arg Met Val Val Asp Gly Ala Met Phe Asn Gln Arg Phe Ile Trp	
145 150 155 160	

Tyr Arg Glu Val Val Asn Tyr Asn Met Pro Lys Glu Val Met Phe Met
 165 170 175
 Ser Ser Val Phe Met Arg Gly Arg His Leu Ile Tyr Leu Arg Tyr Trp
 180 185 190
 Tyr Asp Gly His Val Gly Ser Val Val Pro Ala Met Ser Phe Gly Tyr
 195 200 205
 Ser Ala Leu His Cys Gly Ile Leu Asn Asn Ile Val Val Leu Cys Cys
 210 215 220
 Ser Tyr Cys Ala Asp Leu Ser Glu Ile Arg Val Arg Cys Cys Ala Arg
 225 230 235 240
 Arg Thr Arg Arg Leu Met Leu Arg Ala Val Arg Ile Ile Ala Glu Glu
 245 250 255
 Thr Thr Ala Met Leu Tyr Ser Cys Arg Thr Glu Arg Arg Arg Gln Gln
 260 265 270
 Phe Ile Arg Ala Leu Leu Gln His His Arg Pro Ile Leu Met His Asp
 275 280 285
 Tyr Asp Ser Thr Pro Met
 290

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35408 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: not relevant
 - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATCATCAAT AATATAACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG	50
GGGGTGGAGT TTGTGACGTG GCGCGGGCGC TGGGAACGGG GCGGGTGACG	100
TAGTAGTGTG GCGGAAGTGT GATGTTGCAA GTGTGGCGGA ACACATGTAA	150
GCGACGGATG TGGCAAAAGT GACGTTTTG GTGTGCCCG GTGTACACAG	200
GAAGTGACAA TTTTCGCGCG GTTTAGGCG GATGTTGTAG TAAATTTGGG	250
CGTAACCGAG TAAGATTTGG CCATTTCGC GGGAAACTG AATAAGAGGA	300

AGTGAAATCT	GAATAATTT	GTGTTACTCA	TAGCGCGTAA	TATTTGTCTA	350
GGGAGATCAG	CCTGCAGGTC	GTTACATAAC	TTACGGTAAA	TGGCCCGCCT	400
GGCTGACCGC	CCAACGACCC	CCGCCCATG	ACGTCAATAA	TGACGTATGT	450
TCCCATAGTA	ACGCCAATAG	GGACTTTCCA	TTGACGTCAA	TGGGTGGAGT	500
ATTTACGGTA	AACTGCCAC	TTGGCAGTAC	ATCAAGTGT	TCATATGCCA	550
AGTACGCC	CTATTGACGT	CAATGACGGT	AAATGGCCCG	CCTGGCATT	600
TGCCAGTAC	ATGACCTTAT	GGGACTTCC	TACTTGGCAG	TACATCTACT	650
CGAGGCCACG	TTCTGCTTCA	CTCTCCCCAT	CTCCCCCCCC	TCCCCACCCC	700
CAATTTGTA	TTTATTATT	TTTAATTAT	TTTGTGCAGC	GATGGGGGCG	750
GGGGGGGGGG	GGGGGCGCGC	GCCAGGCAGG	GCAGGGCGGG	GCGAGGGGCG	800
GGGCAGGGCG	AGGCGGAGAG	GTGCGCGGC	AGCCAATCAG	AGCGCGCGC	850
TCCGAAAGTT	TCCTTTATG	GCGAGGCAGC	GGCGGCGGG	GCCCTATAAA	900
AAGCGAAGCG	CGCGGCGGGC	GGGAGCGGG	TCAGCCACCG	CGGTGGCGGC	950
CGCAATTCCC	GGGGATCGAA	AGAGCCTGCT	AAAGAAAAAA	AGAAGTCACC	1000
ATGTCGTTA	CTTGACCAA	CAAGAACGTG	ATTTCGTTG	CCGGTCTGGG	1050
AGGCATTGGT	CTGGACACCA	GCAAGGAGCT	GCTCAAGCGC	GATCCCGTCG	1100
TTTACAACG	TCGTGACTGG	GAAAACCTG	GCCTTACCCA	ACTTAATCGC	1150
CTTGCAGCAC	ATCCCCCTT	CGCCAGCTGG	CGTAATAGCG	AAGAGGCCCG	1200
CACCGATCGC	CCTTCCCAAC	AGTTGCGCAG	CCTGAATGGC	GAATGGCGCT	1250
TTGCCTGGTT	TCCGGCACCA	GAAGCGGTGC	CGGAAAGCTG	GCTGGAGTGC	1300
GATCTTCCTG	AGGCCGATAC	TGTCGTCGTC	CCCTCAAAC	GGCAGATGCA	1350
CGGTTACGAT	GCGCCCATCT	ACACCAACGT	AACCTATCCC	ATTACGGTCA	1400
ATCCGCCGTT	TGTTCCCACG	GAGAATCCGA	CGGGTTGTTA	CTCGCTCACA	1450
TTTAATGTTG	ATGAAAGCTG	GCTACAGGAA	GGCCAGACGC	GAATTATTT	1500
TGATGGCGTT	AACTCGGCAGT	TTCATCTGTG	GTGCAACGGG	CGCTGGGTAG	1550
GTTACGGCCA	GGACAGTCGT	TTGCCGTCTG	AATTTGACCT	GAGCGCATT	1600

TTACGCGCCG	GAGAAAACCG	CCTCGCGGTG	ATGGTGCTGC	GTTGGAGTGA	1650	
CGGCAGTTAT	CTGGAAGATC	AGGATATGTG	GCGGATGAGC	GGCATTTC	1700	
GTGACGTCTC	GTTGCTGCAT	AAACCGACTA	CACAAATCAG	CGATTCCAT	1750	
GTTGCCACTC	GCTTTAATGA	TGATTCAGC	CGCGCTGTAC	TGGAGGCTGA	1800	
AGTTCA	GATG	TGCGGCGAGT	TGCGTGACTA	CCTACGGGTA	ACAGTTCTT	1850
TATGGCAGGG	TGAAACGCAG	GTCGCCAGCG	GCACCGCGCC	TTTCGGCGGT	1900	
GAAATTATCG	ATGAGCGTGG	TGGTTATGCC	GATCGCGTCA	CACTACGTCT	1950	
GAACGTGAA	AAACCGAAAC	TGTGGAGCGC	CGAAATCCCG	AATCTCTATC	2000	
GTGCGGTGGT	TGAAC	TGAC	ACCGCCGACG	GCACGCTGAT	TGAAGCAGAA	2050
GCCTGCGATG	TCGGTTTCCG	CGAGGTGCGG	ATTGAAAATG	GTCTGCTGCT	2100	
GCTGAACGGC	AAGCCGTTGC	TGATTGAGG	CGTTAACCGT	CACGAGCATC	2150	
ATCCTCTGCA	TGGTCAGGTC	ATGGATGAGC	AGACGATGGT	GCAGGATATC	2200	
CTGCTGATGA	AGCAGAACAA	CTTTAACGCC	GTGCGCTGTT	CGCATTATCC	2250	
GAACCATCCG	CTGTGGTACA	CGCTGTGCGA	CCGCTACGGC	CTGTATGTGG	2300	
TGGATGAAGC	CAATATTGAA	ACCCACGGCA	TGGTGCCAAT	GAATCGTCTG	2350	
ACCGATGATC	CGCGCTGGCT	ACCGGCGATG	AGCGAACGCG	TAACGCGAAT	2400	
GGTGCAGCGC	GATCGTAATC	ACCCGAGTGT	GATCATCTGG	TCGCTGGGGA	2450	
ATGAATCAGG	CCACGGCGCT	AATCACGACG	CGCTGTATCG	CTGGATCAA	2500	
TCTGTCGATC	CTTCCC	GGTCAGTAT	GAAGGCAGCG	GAGCCGACAC	2550	
CACGGCCACC	GATATTATTT	GCCCGATGTA	CGCGCGCGTG	GATGAAGACC	2600	
AGCCCTTCCC	GGCTGTGCCG	AAATGGTCCA	TCAAAAAATG	GCTTCGCTA	2650	
CCTGGAGAGA	CGCGCCCCGCT	GATCCTTGC	GAATACGCC	ACCGGATGGG	2700	
TAACAGTCTT	GGCGGTTTCG	CTAAATACTG	GCAGGGCGTT	CGTCAGTATC	2750	
CCCCTTACA	GGGCGGCTTC	GTCTGGACT	GGGTGGATCA	GTGCGCTGATT	2800	
AAATATGATG	AAAACGGCAA	CCCGTGGTCG	GCTTACGGCG	GTGATTTGG	2850	
CGATACGCCG	AACGATCGCC	AGTTCTGTAT	GAACGGTCTG	GTCTTGCCG	2900	

ACCGCACGCC	GCATCCAGCG	CTGACGGAAG	CAAAACACCA	GCAGCAGTTT	2950
TTCCAGTTCC	GTTTATCCGG	GCAAACCATC	GAAGTGACCA	GCGAATACCT	3000
GTTCCGTCAT	ACCGATAACG	AGCTCCTGCA	CTGGATGGTG	GCGCTGGATG	3050
GTAAGCCGCT	GGCAAGCGGT	GAAGTGCCTC	TGGATGTCGC	TCCACAAGGT	3100
AAACAGTTGA	TTGAAC TGCC	TGAAC TACCG	CAGCCGGAGA	GCGCCGGGCA	3150
ACTCTGGCTC	ACAGTACGCG	TAGTGCAACC	GAACGCGACC	GCATGGTCAG	3200
AAGCCGGGCA	CATCAGCGCC	TGGCAGCAGT	GGCGTCTGGC	GGAAAACCTC	3250
AGTGTGACGC	TCCCCGCCGC	GTCCCACGCC	ATCCCGCATC	TGACCACCAG	3300
CGAAATGGAT	TTTGACATCG	AGCTGGTAA	TAAGCGTTGG	CAATTAAACC	3350
GCCAGTCAGG	CTTTCTTTCA	CAGATGTGGA	TTGGCGATAA	AAAACAAC TG	3400
CTGACGCCGC	TGGCGATCA	GTTCACCCGT	GCACCGCTGG	ATAACGACAT	3450
TGGCGTAAGT	GAAGCGACCC	GCATTGACCC	TAACGCCTGG	GTCGAACGCT	3500
GGAAGGCGGC	GGGCCATTAC	CAGGCCGAAG	CAGCGTTGTT	GCAGTGCACG	3550
GCAGATACAC	TTGCTGATGC	GGTGTGATT	ACGACCGCTC	ACGCGTGGCA	3600
GCATCAGGGG	AAAACTTAT	TTATCAGCCG	GAAAACCTAC	CGGATTGATG	3650
GTAGTGGTCA	AATGGCGATT	ACCGTTGATG	TTGAAGTGGC	GAGCGATAACA	3700
CCGCATCCGG	CGCGGATTGG	CCTGAACTGC	CAGCTGGCGC	AGGTAGCAGA	3750
GCGGGTAAAC	TGGCTCGGAT	TAGGGCCGCA	AGAAAAC TAT	CCCGACCGCC	3800
TTACTGCCGC	CTGTTTGAC	CGCTGGGATC	TGCCATTGTC	AGACATGTAT	3850
ACCCC GTACG	TCTTCCCGAG	CGAAAACGGT	CTGCGCTGCG	GGACGCGCGA	3900
ATTGAATTAT	GGCCCACACC	AGTGGCGCGG	CGACTTCCAG	TTCAACATCA	3950
GCCGCTACAG	TCAACAGCAA	CTGATGGAAA	CCAGCCATCG	CCATCTGCTG	4000
CACCGGAAAG	AAGGCACATG	GCTGAATATC	GACGGTTTCC	ATATGGGGAT	4050
TGGTGGCGAC	GACTCCTGGA	GCCC GTCA GT	ATCGGCGGAA	TTACAGCTGA	4100
GCGCCGGTCTG	CTACCATTAC	CAGTTGGTCT	GGTGTCAAAA	ATAATAATAA	4150
CCGGGCAGGC	CATGTCTGCC	CGTATTCGC	GTAAGGAAAT	CCATTATGTA	4200

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ACTTTTTAT CATGGGAGCC TACTTCCCGT TTTTCCCGAT TTGGCTACAT	4300
GACATCAACC ATATCAGCAA AAGTGATACG GGTATTATTT TTGCCGCTAT	4350
TTCTCTGTT CCGCTATTAT TCCAACCGCT GTTTGGTCTG CTTTCTGACA	4400
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CATTGATGAG TTTGGACAAA CCACAACTAG AATGCAGTGA AAAAAATGCT	4500
TTATTTGTGA AATTGTGAT GCTATTGCTT TATTTGTAAAC CATTATAAGC	4550
TGCAATAAAC AAGTTAACAA CAACAATTGC ATTCAATTAA TGTTTCAGGT	4600
TCAGGGGGAG GTGTGGGAGG TTTTTCGGA TCCTCTAGAG TCGACCTGCA	4650
GGCTGATCAG TGGAAGGTGC TGAGGTACGA TGAGACCCGC ACCAGGTGCA	4700
GACCCTGCGA GTGTGGCGGT AAACATATTA GGAACCAGCC TGTGATGCTG	4750
GATGTGACCG AGGAGCTGAG GCCCGATCAC TTGGTGCTGG CCTGCACCCG	4800
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GTGGGCGTGG CTTAAGGGTG GGAAAGAATA TATAAGGTGG GGGTCTTATG	4900
TAGTTTGTA TCTGTTTGC AGCAGCCGCC GCCGCCATGA GCACCAAATC	4950
GTTTGATGGA AGCATTGTGA GCTCATATTT GACAACGCC ATGCCCCAT	5000
GGGCCGGGGT GCGTCAGAAT GTGATGGGCT CCAGCATTGA TGGTCGCC	5050
GTCCTGCCCG CAAACTCTAC TACCTTGACC TACGAGACCG TGTCTGGAAC	5100
GCCGTTGGAG ACTGCAGCCT CCGCCGCCGC TTCAGCCGCT GCAGCCACCG	5150
CCCGCGGGAT TGTGACTGAC TTTGCTTCC TGAGCCCGCT TGCAAGCAGT	5200
GCAGCTTCCC GTTCATCCGC CCGCGATGAC AAGTTGACGG CTCTTTGGC	5250
ACAATTGGAT TCTTGACCC GGGAACTTAA TGTGTTTCT CAGCAGCTGT	5300
TGGATCTGCG CCAGCAGGTT TCTGCCCTGA AGGCTTCCTC CCCTCCCAAT	5350
GCGGTTAAA ACATAAATAA AAAACCAGAC TCTGTTGGA TTTGGATCAA	5400
GCAAGTGTCT TGCTGTCTT ATTTAGGGGT TTTGCGCGCG CGGTAGGCC	5450
GGGACCAGCG GTCTCGGTG TTGAGGGTCC TGTGTATTT TTCCAGGACG	5500

TGGTAAAGGT	GACTCTGGAT	GTCAGATAAC	ATGGGCATAA	GCCCGTCTCT	5550
GGGGTGGAGG	TAGCACCAC	GCAGAGCTTC	ATGCTGCGGG	GTGGTGTGTTGT	5600
AGATGATCCA	GTCGTAGCAG	GAGCGCTGGG	CGTGGTGCT	AAAAATGTCT	5650
TTCAGTAGCA	AGCTGATTGC	CAGGGGCAGG	CCCTTGGTGT	AAGTGTTCAC	5700
AAAGCGGTTA	AGCTGGGATG	GGTGCATAAC	TGGGGATATG	AGATGCATCT	5750
TGGACTGTAT	TTTTAGGTTG	GCTATGTTCC	CAGCCATATC	CCTCCGGGGA	5800
TTCATGTTGT	GCAGAACAC	CAGCACAGTG	TATCCGGTGC	ACTTGGGAAA	5850
TTTGTCATGT	AGCTTAGAAG	GAAATGCGTG	GAAGAACTTG	GAGACGCCCT	5900
TGTGACCTCC	AAGATTTCC	ATGCATTCGT	CCATAATGAT	GGCAATGGGC	5950
CCACGGGCGG	CGGCCTGGC	GAAGATATT	CTGGGATCAC	TAACGTCATA	6000
GTTGTGTTCC	AGGATGAGAT	CGTCATAGGC	CATTTTACA	AAGCGCGGGC	6050
GGAGGGTGCC	AGACTGCGGT	ATAATGGTTC	CATCCGGCCC	AGGGGCGTAG	6100
TTACCCCTCAC	AGATTTGCAT	TTCCCACGCT	TTGAGTTCA	ATGGGGGGAT	6150
CATGTCTACC	TGCGGGGCGA	TGAAGAAAAC	GGTTTCCGGG	GTAGGGGAGA	6200
TCAGCTGGGA	AGAAAGCAGG	TTCCCTGAGCA	GCTGCGACTT	ACCGCAGCCG	6250
GTGGGCCCGT	AAATCACACC	TATTACCGGG	TGCAACTGGT	AGTTAAGAGA	6300
GCTGCAGCTG	CCGTCATCCC	TGAGCAGGGG	GGCCACTTCG	TTAAGCATGT	6350
CCCTGACTCG	CATGTTTCC	CTGACCAAAT	CCGCCAGAAAG	GCGCTCGCCG	6400
CCCAGCGATA	GCAGTTCTTG	CAAGGAAGCA	AAGTTTTCA	ACGGTTTGAG	6450
ACCGTCCGCC	GTAGGCATGC	TTTGAGCGT	TTGACCAAGC	AGTCCAGGC	6500
GGTCCCACAG	CTCGGTCA	TGCTCTACGG	CATCTCGATC	CAGCATATCT	6550
CCTCGTTTCG	CGGGTTGGGG	CGGCTTCGC	TGTACGGCAG	TAGTCGGTGC	6600
TCGTCCAGAC	GGGCCAGGGT	CATGTCTTC	CACGGGCGCA	GGGTCCCTCGT	6650
CAGCGTAGTC	TGGGTACCGG	TGAAGGGGTG	CGCTCCGGGC	TGCGCGCTGG	6700
CCAGGGTGCG	CTTGAGGCTG	GTCCTGCTGG	TGCTGAAGCG	CTGCCGGTCT	6750
TCGCCCTGCG	CGTCGGCCAG	GTAGCATTG	ACCATGGTGT	CATAGTCCAG	6800

CCCCTCCGCG	GCGTGGCCCT	TGGCGCGCAG	CTTGCCTTGT	GAGGAGGC	6850
CGCACGAGGG	GCAGTGCAGA	CTTTGAGGG	CGTAGAGCTT	GGCGCGAGA	6900
AATAACGATT	CCGGGGAGTA	GGCATCCGCG	CCGCAGGCC	CGCAGACGGT	6950
CTCGCATTCC	ACGAGCCAGG	TGAGCTCTGG	CCGTTGGGG	TCAAAACCA	7000
GGTTTCCCCC	ATGCTTTTG	ATGCCTTCT	TACCTCTGGT	TTCCATGAGC	7050
CGGTGTCCAC	GCTCGGTGAC	GAAAAGGCTG	TCCGTGTCCC	CGTATACAGA	7100
CTTGAGAGGC	CTGTCCTCGA	GCGGTGTTCC	GCGGTCTCC	TCGTATAGAA	7150
ACTCGGACCA	CTCTGAGACA	AAGGCTCGCG	TCCAGGCCAG	CACGAAGGAG	7200
GCTAAGTGGG	AGGGGTAGCG	GTCGTTGTCC	ACTAGGGGT	CCACTCGCTC	7250
CAGGGTGTGA	AGACACATGT	CGCCCTCTTC	GGCATCAAGG	AAGGTGATTG	7300
GTTTGTAGGT	GTAGGCCACG	TGACCGGGTG	TTCCCTGAAGG	GGGGCTATAA	7350
AAGGGGGTGG	GGGCGCGTTC	GTCCTCACTC	TCTCCGCAT	CGCTGTCTGC	7400
GAGGGCCAGC	TGTTGGGGTG	AGTACTCCCT	CTGAAAAGCG	GGCATGACTT	7450
CTGCGCTAAG	ATTGTCAGTT	TCCAAAACG	AGGAGGATT	GATATTCA	7500
TGGCCC CGGG	TGATGCCTT	GAGGGTGGCC	GCATCCATCT	GGTCAGAAA	7550
GACAATCTT	TTGTTGTCAA	GCTTGGTGGC	AAACGACCCG	TAGAGGGCGT	7600
TGGACAGCAA	CTTGGCGATG	GAGCGCAGGG	TTTGGTTTT	GTCGCGATCG	7650
GCGCGCTCCT	TGGCCCGCAT	GTTAGCTGC	ACGTATTGCA	GCGCAACGCA	7700
CCGCCATTG	GGAAAGACGG	TGGTGCCTC	GTCGGGCACC	AGGTGCACGC	7750
GCCAACCGCG	GTTGTGCAGG	GTGACAAGGT	CAACGCTGGT	GGCTACCTCT	7800
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CGGTAAAGAC	CCCAGGCAGC	AGGCGCGCGT	CGAAGTAGTC	TATCTGCAT	7950
CCTTGCAAGT	CTAGCGCCTG	CTGCCATGCG	CGGGCGGCAA	GCGCGCGCTC	8000
GTATGGGTTG	AGTGGGGGAC	CCCATGGCAT	GGGGTGGGTG	AGCGCGGAGG	8050
CGTACATGCC	GCAAATGTCG	TAAACGTAGA	GGGGCTCTCT	GAGTATTCCA	8100

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GTATAGTCG	TGCGAGGGAG	CGAGGAGGTC	GGGACCGAGG	TTGCTACGGG	8200
CGGGCTGCTC	TGCTCGGAAG	ACTATCTGCC	TGAAGATGGC	ATGTGAGTTG	8250
GATGATATGG	TTGGACGCTG	GAAGACGTTG	AAGCTGGCGT	CTGTGAGACC	8300
TACCGCGTCA	CGCACGAAGG	AGGCGTAGGA	GTCGCGCAGC	TTGTTGACCA	8350
GCTCGGCCGT	GACCTGCACG	TCTAGGGCGC	AGTAGTCCAG	GGTTTCCTTG	8400
ATGATGTCAT	ACTTATCCTG	TCCCTTTTT	TTCCACAGCT	CGCGGTTGAG	8450
GACAAACTCT	TCGCGGTCTT	TCCAGTACTC	TTGGATCGGA	AACCCGTCGG	8500
CCTCCGAACG	GTAAGAGCCT	AGCATGTAGA	ACTGGTTGAC	GGCCTGGTAG	8550
GCGCAGGCATC	CCTTTCTAC	GGGTAGCGCG	TATGCCTGCG	CGGCCTTCCG	8600
GAGCGAGGTG	TGGGTGAGCG	CAAAGGTGTC	CCTGACCATG	ACTTTGAGGT	8650
ACTGGTATTT	GAAGTCAGTG	TCGTCGCATC	CGCCCTGCTC	CCAGAGCAAA	8700
AAGTCCGTGC	GCTTTTGGA	ACGCGGATTT	GGCAGGGCGA	AGGTGACATC	8750
GTTGAAGAGT	ATCTTCCC	CGCGAGGCAT	AAAGTTGCGT	GTGATGCGGA	8800
AGGGTCCCGG	CACCTCGAA	CGGTTGTTAA	TTACCTGGC	GGCGAGCACG	8850
ATCTCGTCAA	AGCCGTTGAT	GTTGTGGCCC	ACAATGTAAA	GTTCCAAGAA	8900
GCGCGGGATG	CCCTTGATGG	AAGGCAATT	TTAAGTTCC	TCGTAGGTGA	8950
GCTCTTCAGG	GGAGCTGAGC	CCGTGCTCTG	AAAGGGCCCA	GTCTGCAAGA	9000
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TTGCAGGTGG	TCGCGAAAGG	TCCTAAACTG	GCGACCTATG	GCCATTTTT	9100
CTGGGGTGAT	GCAGTAGAAG	GTAAGCGGGT	CTTGTTCCCA	GCGGTCCCCT	9150
CCAAGGTTCG	CGGCTAGGTC	TCGCGCGCA	GTCACTAGAG	GCTCATCTCC	9200
GCCGAACCTTC	ATGACCAGCA	TGAAGGGCAC	GAGCTGCTTC	CCAAAGGCC	9250
CCATCCAAGT	ATAGGTCTCT	ACATCGTAGG	TGACAAAGAG	ACGCTCGGTG	9300
CGAGGGATGCG	AGCCGATCGG	GAAGAACTGG	ATCTCCGCC	ACCAATTGGA	9350
GGAGTGCTA	TTGATGTGGT	GAAAGTAGAA	GTCCCTGCGA	CGGGCCGAAC	9400

ACTCGTGCTG	GCTTTGTAA	AAACGTGCGC	AGTACTGGCA	GCGGTGCACG	9450
GGCTGTACAT	CCTGCACGAG	GTTGACCTGA	CGACCGCGCA	CAAGGAAGCA	9500
GAGTGGGAAT	TTGAGCCCC	CGCCTGGCGG	TTTGGCTGG	TGGTCTTCTA	9550
CTTCGGCTGC	TTGTCCCTGA	CCGTCTGGCT	GCTCGAGGGG	AGTTACGGTG	9600
GATCGGACCA	CCACGCCGCG	CGAGCCCCAA	GTCCAGATGT	CCGCGCGCGG	9650
CGGTGGAGC	TTGATGACAA	CATCGCGCAG	ATGGGAGCTG	TCCATGGTCT	9700
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CATAGACGGG	TCAGGGCGCG	GGCTAGATCC	AGGTGATACC	TAATTTCCAG	9800
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GCGCGACTAC	GGTACCGCGC	GGCGGGCGGT	GGGCCGCGGG	GGTGTCTTG	9900
GATGATGCAT	CTAAAAGCGG	TGACGCGGGC	GAGCCCCCGG	AGGTAGGGGG	9950
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GGGCAGGAGC	TGGTGCTGCG	CGCGTAGGTT	GCTGGCGAAC	GCGACGACGC	10050
GGCGGTTGAT	CTCCTGAATC	TGGCGCCTCT	GCGTGAAGAC	GACGGGCCCC	10100
GTGAGCTTGA	GCCTGAAAGA	GAGTCGACA	GAATCAATT	CGGTGTGTT	10150
GACGGCGGCC	TGGCGAAAAA	TCTCCTGCAC	GTCTCCTGAG	TTGTCTTGAT	10200
AGGCGATCTC	GGCCATGAAC	TGCTCGATCT	CTTCCTCCTG	GAGATCTCCG	10250
CGTCCGGCTC	GCTCCACGGT	GGCGGCGAGG	TCGTTGGAAA	TGCGGGCCAT	10300
GAGCTGCGAG	AAGGCAGTTGA	GGCCTCCCTC	GTTCCAGACG	CGGCTGTAGA	10350
CCACGCCCCC	TTCGGCATCG	CGGGCGCGCA	TGACCACCTG	CGCGAGATTG	10400
AGCTCCACGT	GCCGGGCGAA	GACGGCGTAG	TTTCGCAGGC	GCTGAAAGAG	10450
GTAGTTGAGG	GTGGTGGCGG	TGTGTTCTGC	CACGAAGAAC	TACATAACCC	10500
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TCGCGAGACT	GGGGCGTAC	ACTGGATGGC	CTTTGCCTGG	AACCCGCACT	23250
CAAAAACATG	CTACCTCTTT	GAGCCCTTG	GCTTTCTGA	CCAGCGACTC	23300
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TGCTTCTTCC	CCCGACCGCT	GTATAACGCT	GGAAAAGTCC	ACCCAAAGCG	23400
TACAGGGGCC	CAACTCGGCC	GCCTGTGGAC	TATTCTGCTG	CATGTTCTC	23450
CACGCCTTG	CCAACTGGCC	CCAAACTCCC	ATGGATCACA	ACCCCACCAT	23500
GAACCTTATT	ACCGGGGTAC	CCAACTCCAT	GCTCAACAGT	CCCCAGGTAC	23550
AGCCCACCCCT	GGTCGCAAC	CAGGAACAGC	TCTACAGCTT	CCTGGAGCGC	23600
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TTTTGTCAC	TTGAAAAACA	TGTAAAATA	ATGTACTAGA	GACACTTTCA	23700

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ACCCCTGCCG	TCTGCGCCGT	TTAAAAATCA	AAGGGGTTCT	GCCGCGCATC	23800
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100

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101

TCCACCCCT CATTATCATA TTGGCTTCAA TCCAAAATAA GGTATATTAT	35400
TGATGATG	35408

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8509 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: not relevant

- (ii) MOLECULE TYPE: cDNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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102

TCTTTTGTC	TTTTATTCA	GGTCCCGGAT	CCGGTGGTGG	TGCAAATCAA	950
AGAAACTGCTC	CTCAGTGGAT	GTTGCCTTA	CTTCTAGGCC	TGTACGGAAG	1000
TGTTACTTCT	GCTCTAAAAG	CTGCGGAATT	GTACCCGCGG	CCGCAATTCC	1050
CGGGGATCGA	AAGAGCCTGC	TAAAGCAAAA	AAGAAGTCAC	CATGTCGTTT	1100
ACTTTGACCA	ACAAGAACGT	GATTTCGTT	GCCGGTCTGG	GAGGCATTGG	1150
TCTGGACACC	AGCAAGGAGC	TGCTCAAGCG	CGATCCCCTC	GTTTACAAC	1200
GTCGTGACTG	GGAAAACCT	GGCGTTACCC	AACTTAATCG	CCTTGCAGCA	1250
CATCCCCCTT	TCGCCAGCTG	GCGTAATAGC	GAAGAGGCC	GCACCGATCG	1300
CCCTTCCCAA	CAGTTGCGCA	GCCTGAATGG	CGAATGGCGC	TTTGCCTGGT	1350
TTCCGGCACC	AGAAGCGGTG	CCGGAAAGCT	GGCTGGAGTG	CGATCTTCCT	1400
GAGGCCGATA	CTGTCGTCGT	CCCCTCAAAC	TGGCAGATGC	ACGGTTACGA	1450
TGCGCCCATC	TACACCAACG	TAACCTATCC	CATTACGGTC	AATCCGCCGT	1500
TTGTTCCCAC	GGAGAATCCG	ACGGGTTGTT	ACTCGCTCAC	ATTTAATGTT	1550
GATGAAAGCT	GGCTACAGGA	AGGCCAGACG	CGAATTATTT	TTGATGGCGT	1600
TAACTCGGCG	TTTCATCTGT	GGTGCAACGG	GCGCTGGGTC	GGTTACGGCC	1650
AGGACAGTCG	TTTGCCTCT	GAATTTGACC	TGAGCGCATT	TTTACGCGCC	1700
GGAGAAAACC	GCCTCGCGGT	GATGGTGCTG	CGTTGGAGTG	ACGGCAGTTA	1750
TCTGGAAGAT	CAGGATATGT	GGCGGATGAG	CGGCATTTTC	CGTGACGTCT	1800
CGTTGCTGCA	TAAACCGACT	ACACAAATCA	GCGATTCCA	TGTTGCCACT	1850
CGCTTTAATG	ATGATTTCA	CCCGCGCTGTA	CTGGAGGCTG	AAGTTCAAGAT	1900
GTGCGGCGAG	TTGCGTGA	ACCTACGGGT	AACAGTTCT	TTATGGCAGG	1950
GTGAAACGCA	GGTCGCCAGC	GGCACCGCGC	CTTCCGGCGG	TGAAATTATC	2000
GATGAGCGTG	GTGGTTATGC	CGATCGCGTC	ACACTACGTC	TGAACGTCGA	2050
AAACCCGAAA	CTGTGGAGCG	CCGAAATCCC	GAATCTCTAT	CGTGCAGGTGG	2100
TTGAACTGCA	CACCGCCGAC	GGCACGCTGA	TTGAAGCAGA	AGCCTGCGAT	2150
GTCGGTTTCC	GCGAGGTGCG	GATTGAAAAT	GGTCTGCTGC	TGCTGAACGG	2200

CAAGCCGTTG	CTGATTCGAG	GCGTTAACCG	TCACGAGCAT	CATCCTCTGC	2250
ATGGTCAGGT	CATGGATGAG	CAGACGATGG	TGCAGGATAT	CCTGCTGATG	2300
AAGCAGAACAA	ACTTTAACGC	CGTGCCTGT	TCGCATTATC	CGAACCATCC	2350
GCTGTGGTAC	ACGCTGTGCG	ACCGCTACGG	CCTGTATGTG	GTGGATGAAG	2400
CCAATATTGA	AACCCACGGC	ATGGTGCCAA	TGAATCGTCT	GACCGATGAT	2450
CCGCGCTGGC	TACCGGCGAT	GAGCGAACGC	GTAACGCGAA	TGGTGCAGCG	2500
CGATCGTAAT	CACCCGAGTG	TGATCATCTG	GTCGCTGGGG	AATGAATCAG	2550
GCCACGGCGC	TAATCACGAC	GCGCTGTATC	GCTGGATCAA	ATCTGTCGAT	2600
CCTTCCCGCC	CGGTGCAGTA	TGAAGGCGGC	GGAGCCGACA	CCACGGCCAC	2650
CGATATTATT	TGCCCGATGT	ACGCGCGCGT	GGATGAAGAC	CAGCCCTTCC	2700
CGGCTGTGCC	GAAATGGTCC	ATCAAAAAAT	GGCTTCGCT	ACCTGGAGAG	2750
ACGCGCCCGC	TGATCCTTG	CGAATACGCC	CACCGATGG	GTAACAGTCT	2800
TGGCGGTTTC	GCTAAATACT	GGCAGGCAGT	TCGTCAGTAT	CCCCGTTTAC	2850
AGGGCGGCTT	CGTCTGGGAC	TGGGTGGATC	AGTCGCTGAT	TAAATATGAT	2900
GAAAACGGCA	ACCCGTGGTC	GGCTTACGGC	GGTGATTTG	GCGATACGCC	2950
GAACGATCGC	CAGTTCTGTA	TGAACGGTCT	GGTCTTGCC	GACCGCACGC	3000
CGCATCCAGC	GCTGACGGAA	GCAAAACACC	AGCAGCAGTT	TTTCCAGTTC	3050
CGTTTATCCG	GGCAAACCAT	CGAAGTGACC	AGCGAATACC	TGTTCCGTCA	3100
TAGCGATAAC	GAGCTCCTGC	ACTGGATGGT	GGCGCTGGAT	GGTAAGCCGC	3150
TGGCAAGCGG	TGAAGTGCCT	CTGGATGTCG	CTCCACAAAGG	TAAACAGTTG	3200
ATTGAACTGC	CTGAACCTACC	GCAGCCGGAG	AGCGCCGGGC	AACTCTGGCT	3250
CACAGTACGC	GTAGTGCAC	CGAACGCGAC	CGCATGGTCA	GAAGCCGGGC	3300
ACATCAGCGC	CTGGCAGCAG	TGGCGTCTGG	CGGAAAACCT	CAGTGTGACG	3350
CTCCCCGCCG	CGTCCCACGC	CATCCCGCAT	CTGACCACCA	GCGAAATGGA	3400
TTTTTGCATC	GAGCTGGGTA	ATAAGCGTTG	GCAATTAAAC	CGCCAGTCAG	3450
GCTTTCTTTC	ACAGATGTGG	ATTGGCGATA	AAAAACAACT	GCTGACGCCG	3500

104

CTGCGCGATC AGTTCACCCG TGCACCGCTG GATAACGACA TTGGCGTAAG	3550
TGAAGCGACC CGCATTGACC CTAACGCCTG GGTCGAACGC TGGAAGGCAG	3600
CGGGCCATTA CCAGGCCGAA GCAGCGTTGT TGCAAGTGCAC GGCAGATAACA	3650
CTTGCTGATG CGGTGCTGAT TACGACCGCT CACCGGTGGC AGCATCAGGG	3700
GAAAACCTTA TTTATCAGCC GGAAAACCTA CCGGATTGAT GGTAGTGGTC	3750
AAATGGCGAT TACCGTTGAT GTTGAAGTGG CGAGCGATAC ACCGCATCCG	3800
GCGCGGATTG GCCTGAACTG CCAGCTGGCG CAGGTAGCAG AGCGGGTAAA	3850
CTGGCTCGGA TTAGGGCCGC AAGAAAACCTA TCCCGACCGC CTTACTGCCG	3900
CCTGTTTGA CCGCTGGGAT CTGCCATTGT CAGACATGTA TACCCCGTAC	3950
GTCTTCCCGA GCGAAAACGG TCTGCGCTGC GGGACGCGCG AATTGAATTA	4000
TGGCCCACAC CAGTGGCGCG GCGACTTCCA GTTCAACATC AGCCGCTACA	4050
GTCAACAGCA ACTGATGGAA ACCAGCCATC GCCATCTGCT GCACGCGGAA	4100
GAAGGCACAT GGCTGAATAT CGACGGTTTC CATATGGGA TTGGTGGCGA	4150
CGACTCCTGG AGCCCCTCAG TATCGGCGGA ATTACAGCTG AGCGCCGGTC	4200
GCTACCATTA CCAGTTGGTC TGGTGTCAAA AATAATAATA ACCGGGCAGG	4250
CCATGTCTGC CCGTATTCG CGTAAGGAAA TCCATTATGT ACTATTTAAA	4300
AAACACAAAC TTTGGATGT TCGGTTTATT CTTTTCTTT TACTTTTTA	4350
TCATGGGAGC CTACTTCCCG TTTTCCCGA TTTGGCTACA TGACATCAAC	4400
CATATCAGCA AAAGTGATAC GGGTATTATT TTTGCCGCTA TTTCTCTGTT	4450
CTCGCTATTA TTCCAACCGC TGTTGGTCT GCTTCTGAC AAACCTGGCC	4500
TCGACTCTAG GCGGCCGCGG GGATCCAGAC ATGATAAGAT ACATTGATGA	4550
GTTTGGACAA ACCACAACTA GAATGCAGTG AAAAAAATGC TTTATTGTG	4600
AAATTTGTGA TGCTATTGCT TTATTTGTAA CCATTATAAG CTGCAATAAA	4650
CAAGTTAACCA ACAACAATTG CATTCAATTG ATGTTTCAGG TTCAGGGGGA	4700
GGTGTGGGAG GTTTTTCCGG ATCCTCTAGA GTCGACCTGC AGGGGCTAGA	4750
ATGGCTACGT AGATAAGTAG CATGGCGGGT TAATCATTAA CTACAAGGAA	4800

105

CCCCTAGTGA	TGGAGTTGGC	CACTCCCTCT	CTGCGCGCTC	GCTCGCTCAC	4850
TGAGGCCGGG	CGACCAAAGG	TCGCCCAGCG	CCCGGGCTTT	GCCCCGGCGG	4900
CCTCAGTGAG	CGAGCGAGCG	CGCAGCTGGC	GTAATAGCGA	AGAGGCCCGC	4950
ACCGATCGCC	CTTCCCAACA	GTTGCGCAGC	CTGAATGGCG	AATGGAATT	5000
CAGACGATTG	AGCGTCAAAA	TGTAGGTATT	TCCATGAGCG	TTTTCCCTGT	5050
TGCAATGGCT	GGCGGTAATA	TTGTTCTGGA	TATTACCAGC	AAGGCCGATA	5100
GTGGAGTTC	TTCTACTCAG	GCAAGTGATG	TTATTACTAA	TCAAAGAAGT	5150
ATTGCGACAA	CGGTTAATT	GCGTGATGGA	CAGACTCTT	TACTCGGTGG	5200
CCTCACTGAT	TATAAAACA	CTTCTCAGGA	TTCTGGCGTA	CCGTTCCCTGT	5250
CTAAAATCCC	TTTAATCGGC	CTCCTGTTA	GCTCCCGCTC	TGATTCTAAC	5300
GAGGAAAGCA	CGTTATACGT	GCTCGTCAAA	GCAACCATAG	TACGCCGCCT	5350
GTAGCGGCGC	ATTAAGCGCG	GCGGGTGTGG	TGGTTACGCG	CAGCGTGACC	5400
GCTACACTTG	CCAGCGCCCT	AGCGCCCGCT	CCTTCGCTT	TCTTCCCTTC	5450
CTTTCTCGCC	ACGTTCGCCG	GCTTCCCCG	TCAAGCTCTA	AATCGGGGGC	5500
TCCCTTTAGG	GTTCCGATTT	AGTGCTTAC	GGCACCTCGA	CCCCAAAAAA	5550
CTTGATTAGG	GTGATGGTTC	ACGTAGTGGG	CCATCGCCCT	GATAGACGGT	5600
TTTCGCCCT	TTGACGTTGG	AGTCCACGTT	CTTTAATAGT	GGACTCTTGT	5650
TCCAAACTGG	AACAACACTC	AACCCTATCT	CGGTCTATT	TTTGATT	5700
TAAGGGATTT	TGCCGATTT	GGCCTATTGG	TTAAAAAAATG	AGCTGATT	5750
ACAAAAAATT	AACGCGAATT	TTAACAAAAT	ATTAACGTT	ACAATT	5800
TATTTGCTTA	TACAATCTTC	CTGTTTTGG	GGCTTTCTG	ATTATCAACC	5850
GGGGTACATA	TGATTGACAT	GCTAGTTTA	CGATTACCGT	TCATCGATT	5900
TCTTGTTC	TCCAGACTCT	CAGGCAATGA	CCTGATAGCC	TTTGTAGAGA	5950
CCTCTAAAAA	ATAGCTACCC	TCTCCGGCAT	GAATTTATCA	GCTAGAACGG	6000
TTGAATATCA	TATTGATGGT	GATTGACTG	TCTCCGGCCT	TTCTCACCCG	6050
TTTGAATCTT	TACCTACACA	TTACTCAGGC	ATTGCATT	AAATATATGA	6100

GGGTTCTAAA	AATTTTATC	CTTGCCTTGA	AATAAAGGCT	TCTCCCGCAA	6150
AAGTATTACA	GGGTCTATAAT	GTTCCTGGTA	CAACCGATTT	AGCTTTATGC	6200
TCTGAGGCTT	TATTGCTTAA	TTTGCTAAT	TCTTGCCTT	GCCTGTATGA	6250
TTTATTGGAT	GTTGGAATTTC	CTGATGCCTT	ATTTCTCCT	TACGCATCTG	6300
TGCCGTATTT	CACACCGCAT	ATGGTGCACT	CTCAGTACAA	TCTGCTCTGA	6350
TGCCGCATAG	TTAAGCCAGC	CCCGACACCC	GCCAACACCC	GCTGACGCGC	6400
CCTGACGGGC	TTGTCTGCTC	CCGGCATCCG	CTTACAGACA	AGCTGTGACC	6450
GTCTCCGGGA	GCTGCATGTG	TCAGAGGTTT	TCACCGTCAT	CACCGAAACG	6500
CGCGAGACGA	AAGGGCCTCG	TGATACGCCT	ATTTTATAG	GTAAATGTCA	6550
TGATAATAAT	GGTTTCTTAG	ACGTCAGGTG	GCACTTTCG	GGGAAATGTG	6600
CGCGGAACCC	CTATTTGTTT	ATTTTCTAA	ATACATTCAA	ATATGTATCC	6650
GCTCATGAGA	CAATAACCCCT	GATAAATGCT	TCAATAATAT	TGAAAAAGGA	6700
AGAGTATGAG	TATTCAACAT	TTCCGTGTCG	CCCTTATTCC	CTTTTTGCG	6750
GCATTTGCC	TTCCTGTTTT	TGCTCACCCA	GAAACGCTGG	TGAAAGTAAA	6800
AGATGCTGAA	GATCAGTTGG	GTGCACGAGT	GGGTTACATC	GAACGGATC	6850
TCAACAGCGG	TAAGATCCTT	GAGAGTTTC	GCCCCGAAGA	ACGTTTTCCA	6900
ATGATGAGCA	CTTTTAAAGT	TCTGCTATGT	GGCGCGGTAT	TATCCCGTAT	6950
TGACGCCGGG	CAAGAGCAAC	TCGGTCGCCG	CATACACTAT	TCTCAGAATG	7000
ACTTGGTTGA	GTACTCACCA	GTCACAGAAA	AGCATCTTAC	GGATGGCATG	7050
ACAGTAAGAG	AATTATGCAG	TGCTGCCATA	ACCATGAGTG	ATAACACTGC	7100
GGCCAACCTTA	CTTCTGACAA	CGATCGGAGG	ACCGAAGGAG	CTAACCGCTT	7150
TTTGCACAA	CATGGGGGAT	CATGTAACTC	GCCTTGATCG	TTGGGAACCG	7200
GAGCTGAATG	AAGCCATACC	AAACGACGAG	CGTGACACCA	CGATGCCTGT	7250
AGCAATGGCA	ACAACGTTGC	GCAAACTATT	AACTGGCGAA	CTACTTACTC	7300
TAGCTTCCCG	GCAACAATTA	ATAGACTGGA	TGGAGGCGGA	TAAAGTTGCA	7350
GGACCACTTC	TGGCGCTCGGC	CCTTCCGGCT	GGCTGGTTA	TTGCTGATAA	7400

107

ATCTGGAGCC	GGTGAGCGTG	GGTCTCGCGG	TATCATTGCA	GCAC TGGGGC	7450
CAGATGGTAA	GCCCTCCCGT	ATCGTAGTTA	TCTACACGAC	GGGGAGTCAG	7500
GCAACTATGG	ATGAACGAAA	TAGACAGATC	GCTGAGATAG	GTGCCTCACT	7550
GATTAAGCAT	TGGTAACTGT	CAGACCAAGT	TTACTCATAT	ATACTTTAGA	7600
TTGATTTAAA	ACTTCATTTC	TAATTTAAAA	GGATCTAGGT	GAAGATCCTT	7650
TTTGATAATC	TCATGACCAA	AATCCCTTAA	CGTGAGTTT	CGTTCCACTG	7700
AGCGTCAGAC	CCC GTAGAAA	AGATCAAAGG	ATCTTCTTGA	GATCCTTTT	7750
TTCTGCGCGT	AATCTGCTGC	TTGCAAACAA	AAAAACCACC	GCTACCAGCG	7800
GTGGTTGTT	TGCCGGATCA	AGAGCTACCA	ACTCTTTTC	CGAAGGTAAC	7850
TGGCTTCAGC	AGAGCGCAGA	TACCAAATAC	TGTCCTTCTA	GTGTAGCCGT	7900
AGTTAGGCCA	CCACTTCAAG	AACTCTGTAG	CACCGCCTAC	ATACCTCGCT	7950
CTGCTAATCC	TGTTACCAGT	GGCTGCTGCC	AGTGGCGATA	AGTCGTGTCT	8000
TACCGGGTTG	GACTCAAGAC	GATA GTTACC	GGATAAGGCG	CAGCGGTCGG	8050
GCTGAACGGG	GGGTTCGTGC	ACACAGCCCA	GCTTGGAGCG	AACGACCTAC	8100
ACCGAACTGA	GATA CCTACA	GGTGAGCTA	TGAGAAAGCG	CCACGCTTCC	8150
CGAAGGGAGA	AAGGCGGACA	GGTATCCGGT	AAGCGGCAGG	GT CGGAACAG	8200
GAGAGCGCAC	GAGGGAGCTT	CCAGGGGAA	ACGCCTGGTA	TCTTATAGT	8250
CCTGTCGGGT	TTCGCCACCT	CTGACTTGAG	CGTCGATTT	TGTGATGCTC	8300
GTCAGGGGGG	CGGAGCCTAT	GGAAAAACGC	CAGCAACGCG	GCCTTTTAC	8350
GGTTCCCTGGC	CTTTTGCTGG	CCTTTGCTC	ACATGTTCTT	TCCTGCGTTA	8400
TCCCCTGATT	CTGTGGATAA	CCGTATTACC	GCCTTGAGT	GAGCTGATAC	8450
CGCTCGCCGC	AGCCGAACGA	CCGAGCGCAG	CGAGTCAGTG	AGCGAGGAAG	8500
CGGAAGAGC					8509

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8299 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCCAATAACG	CAAACCGCCT	CTCCCCGCGC	GTTGGCCGAT	TCATTAATGC	50
AGCTGCGCGC	TCGCTCGCTC	ACTGAGGCCG	CCCGGGCAAA	GCCCGGGCGT	100
CGGGCGACCT	TTGGTCGCCC	GGCCTCAGTG	AGCGAGCGAG	CGCGCAGAGA	150
GGGAGTGGCC	AACTCCATCA	CTAGGGGTTTC	CTTGTAGTTA	ATGATTAACC	200
CGCCATGCTA	CTTATCTACA	TCATCGATGA	ATTGAGCTT	GCATGCCTGC	250
AGGTCGTTAC	ATAACTTACG	GTAAATGCC	CGCCTGGCTG	ACCGCCCAAC	300
GACCCCCGCC	CATTGACGTC	AATAATGACG	TATGTTCCA	TAGTAACGCC	350
AATAGGGACT	TTCCATTGAC	GTCAATGGGT	GGAGTATTAA	CGGTAAACTG	400
CCCACTTGGC	AGTACATCAA	GTGTATCATA	TGCCAAGTAC	GCCCCCTATT	450
GACGTCAATG	ACGGTAAATG	GCCCGCCTGG	CATTATGCC	AGTACATGAC	500
CTTATGGGAC	TTTCCTACTT	GGCAGTACAT	CTACGTATTA	GTCATCGCTA	550
TTACCATGGT	GATGCGGTTT	TGGCAGTACA	TCAATGGCG	TGGATAGCGG	600
TTTGACTCAC	GGGGATTTCC	AAGTCTCCAC	CCCATTGACG	TCAATGGGAG	650
TTTGTGTTGG	CACCAAAATC	AACGGGACTT	TCCAAAATGT	CGTAACAACT	700
CCGCCCCATT	GACGCAAATG	GGCGGTAGGC	GTGTACGGTG	GGAGGTCTAT	750
ATAAGCAGAG	CTCGTTAGT	GAACCGTCAG	ATCGCCTGGA	GACGCCATCC	800
ACGCTGTTT	GACCTCCATA	GAAGACACCG	GGACCGATCC	AGCCTCCGGA	850
CTCTAGAGGA	TCCGGTACTC	GACCCGAGCT	CGGATCCACT	AGTAACGGCC	900
GCCAGTGTGC	TGGAATTCTG	CACTCCAGGC	TGCCCGGGTT	TGCATGCTGC	950
TGCTGCTGCT	GCTGCTGGC	CTGAGGCTAC	AGCTCTCCCT	GGGCATCATC	1000

109

CTAGTTGAGG	AGGAGAAACCC	GGACTTCTGG	AACCGCGAGG	CAGCCGAGGC	1050
CCTGGGTGCC	GCCAAGAACG	TGCAGCCTGC	ACAGACAGCC	GCCAAGAACCC	1100
TCATCATCTT	CCTGGGCGAT	GGGATGGGGG	TGTCTACGGT	GACAGCTGCC	1150
AGGATCCTAA	AAGGGCAGAA	GAAGGACAAA	CTGGGGCCTG	AGATAACCCCT	1200
GGCCATGGAC	CGCTTCCCCT	ATGTGGCTCT	GTCCAAGACA	TACAATGTAG	1250
ACAAACATGT	GCCAGACAGT	GGAGCCACAG	CCACGGCCTA	CCTGTGCGGG	1300
GTCAAGGGCA	ACTTCCAGAC	CATTGGCTTG	AGTGCAGCCG	CCCGCTTTAA	1350
CCAGTGCAAC	ACGACACGCG	GCAACGAGGT	CATCTCCGTG	ATGAATCGGG	1400
CCAAGAAAGC	AGGGAAGTCA	GTGGGAGTGG	TAACCACCAC	ACGAGTGCAG	1450
CACGCCCTCGC	CAGCCGGCAC	CTACGCCAC	ACGGTGAACC	GCAACTGGTA	1500
CTCGGACGCC	GACGTGCCCTG	CCTCGGCCCCG	CCAGGAGGGG	TGCCAGGACA	1550
TCGCTACGCA	GCTCATCTCC	AACATGGACA	TTGATGTGAT	CCTAGGTGGA	1600
GGCCGAAAGT	ACATGTTTCG	CATGGGAACC	CCAGACCCCTG	AGTACCCAGA	1650
TGACTACAGC	CAAGGTGGGA	CCAGGCTGGA	CGGGAAAGAAT	CTGGTGCAGG	1700
AATGGCTCGG	CGAACGCCAG	GGTGCCCCGT	ACGTGTGGAA	CCGCACTGAG	1750
CTCATGCAGG	CTTCCCTGGA	CCCGTCTGTG	ACCCATCTCA	TGGGTCTCTT	1800
TGAGCCTGGA	GACATGAAAT	ACGAGATCCA	CCGAGACTCC	ACACTGGACC	1850
CCTCCCTGAT	GGAGATGACA	GAGGCTGCC	TGCGCCTGCT	GAGCAGACAC	1900
CCCCGCGGCT	TCTTCCTCTT	CGTGGAGGGT	GGTCGCATCG	ACCATGGTCA	1950
TCATGAAAGC	AGGGCTTACC	GGCACTGAC	TGAGACGATC	ATGTTCGACG	2000
ACGCCATTGA	GAGGGCGGGC	CAGCTCACCA	GCGAGGAGGA	CACGCTGAGC	2050
CTCGTCACTG	CCGACCACTC	CCACGTCTTC	TCCTTCGGAG	GCTACCCCT	2100
GCGAGGGAGC	TCCTTCATCG	GGCTGGCCGC	TGGCAAGGCC	CGGGACAGGA	2150
AGGCCTACAC	GGTCCTCCTA	TACGGAAACG	GTCCAGGCTA	TGTGCTCAAG	2200
GACGGCGCCC	GGCCGGATGT	TACCGAGAGC	GAGAGCGGGA	GCCCCGAGTA	2250
TCGGCAGCAG	TCAGCAGTGC	CCCTGGACGA	AGAGACCCAC	GCAGGCGAGG	2300

110

ACGTGGCGGT	GTTCGCGCGC	GGCCCGCAGG	CGCACCTGGT	TCACGGCGTG	2350
CAGGAGCAGA	CCTTCATAGC	GCACGTCATG	GCCTTCGCCG	CCTGCCTGGA	2400
GCCCTACACC	GCCTGCGACC	TGGCGCCCCC	CGCCGGCACC	ACCGACGCCG	2450
CGCACCCGGG	GCGGTCCGTG	GTCCCCGCGT	TGCTTCCTCT	GCTGGCCGGG	2500
ACCCCTGCTGC	TGCTGGAGAC	GGCCACTGCT	CCCTGAGTGT	CCCGTCCCTG	2550
GGGCTCCTGC	TTCCCCATCC	CGGAGTTCTC	CTGCTCCCCA	CCTCCTGTG	2600
TCCTGCCTGG	CCTCCAGCCC	GAGTCGTAT	CCCCGGAGTC	CCTATAACAGA	2650
GGTCCTGCCA	TGGAACCTTC	CCCTCCCCGT	GCGCTCTGGG	GACTGAGCCC	2700
ATGACACCAA	ACCTGCCCC	TGGCTGCTCT	CGGACTCCCT	ACCCCAACCC	2750
CAGGGACTGC	AGGTTGTGCC	CTGTGGCTGC	CTGCACCCCA	GGAAAGGAGG	2800
GGGCTCAGGC	CATCCAGCCA	CCACCTACAG	CCCAGTGGGG	TCGAGACAGA	2850
TGGTCAGTCT	GGAGGATGAC	GTGGCGTGAA	GCTGGCCGCG	GGGATCCAGA	2900
CATGATAAGA	TACATTGATG	AGTTTGGACA	AACCACAACT	AGAATGCAGT	2950
GAAAAAAATG	CTTTATTTGT	GAAATTGTG	ATGCTATTGC	TTTATTTGTA	3000
ACCATTATAA	GCTGCAATAA	ACAAGTTAAC	AACAACAATT	GCATTCAATT	3050
TATGTTTCAG	GTTCAGGGGG	AGGTGTGGGA	GGTTTTTCG	GATCCTCTAG	3100
AGTCGACTCT	AGANNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3150
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3200
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3250
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3300
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3350
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNGGATCCC	3400
CATGACTACG	TCCGGCGTTC	CATTTGGCAT	GACACTACGA	CCAACACGAT	3450
CTCGGTTGTC	TCGGCGCACT	CCGTACAGTA	GGGATCGTCT	ACCTCCTTT	3500
GAGACAGAAA	CCCGCGCTAC	CATACTGGAG	GATCATCCGC	TGCTGCCCGA	3550
ATGTAACACT	TTGACAATGC	ACAACGTGAG	TTACGTGCGA	GGTCTTCCCT	3600

111

GCAGTGTGGG	ATTACGCTG	ATTCAGGAAT	GGGTTGTTCC	CTGGGATATG	3650
GTTCTAACGC	GGGAGGAGCT	TGTAATCCTG	AGGAAGTGT	TGCACGTGTG	3700
CCTGTGTGT	GCCAACATTG	ATATCATGAC	GAGCATGATG	ATCCATGGTT	3750
ACGAGTCCTG	GGCTCTCCAC	TGTCATTGTT	CCAGTCCCGG	TTCCCTGCAG	3800
TGTATAGCCG	GCGGGCAGGT	TTTGGCCAGC	TGGTTTAGGA	TGGTGGTGGA	3850
TGGCGCCATG	TTTAATCAGA	GGTTTATATG	GTACCGGGAG	GTGGTGAATT	3900
ACAACATGCC	AAAAGAGGTA	ATGTTTATGT	CCAGCGTGT	TATGAGGGGT	3950
CGCCACTTAA	TCTACCTGCG	CTTGTGGTAT	GATGGCCACG	TGGGTTCTGT	4000
GGTCCCCGCC	ATGAGCTTG	GATACAGCGC	CTTGCACTGT	GGGATTTGA	4050
ACAATATTGT	GGTGCTGTGC	TGCAGTTACT	GTGCTGATT	AAGTGAGATC	4100
AGGGTGCCTG	GCTGTGCCCG	GAGGACAAGG	CGCCTTATGC	TGCGGGCGGT	4150
GCGAATCATC	GCTGAGGAGA	CCACTGCCAT	GTTGTATTCC	TGCAGGACGG	4200
AGCGGCGGCG	GCAGCAGTTT	ATTCGCGCGC	TGCTGCAGCA	CCACCGCCCT	4250
ATCCTGATGC	ACGATTATGA	CTCTACCCCC	ATGTAGGGAT	CCCCATCACT	4300
AGTGCGCCCG	CGGGGATCCA	GACATGATAA	GATACATTGA	TGAGTTTGGA	4350
CAAACCACAA	CTAGAATGCA	GTGAAAAAAA	TGCTTATT	GTGAAATTG	4400
TGATGCTATT	GCTTATTG	TAACCATTAT	AAGCTGCAAT	AAACAAGTTA	4450
ACAACAACAA	TTGCATTCA	TTTATGTTTC	AGGTTCAGGG	GGAGGTGTGG	4500
GAGGTTTTT	CGGATCCTCT	AGAGTCGACC	TGCAGGCATG	CAAGCTGTAG	4550
ATAAGTAGCA	TGGCGGGTTA	ATCATTAACT	ACAAGGAACC	CCTAGTGATG	4600
GAGTTGCCA	CTCCCTCTCT	GCGCGCTCGC	TCGCTCACTG	AGGCCGGCG	4650
ACCAAAGGTC	GCCCGACGCC	CGGGCTTGC	CCGGGCGGCC	TCAGTGAGCG	4700
AGCGAGCGCG	CAGCTGGCGT	AATAGCGAAG	AGGCCCGCAC	CGATGCCCT	4750
TCCCAACAGT	TGCGCAGCCT	GAATGGCGAA	TGGAANTTCC	AGACGATTGA	4800
GCGTAAAAT	GTAGGTATT	CCATGAGCGT	TTTCCTGTT	GCAATGGCTG	4850
GCAGTAATAT	TGTTCTGGAT	ATTACCAAGCA	AGGCCGATAG	TTTGAGTTCT	4900

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114

CCCGTAGAAA	AGATCAAAGG	ATCTTCTTGA	GATCCTTTT	TTCTGCGCGT	7550
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AGCCGAACGA	CCGAGCGCAG	CGAGTCAGTG	AGCGAGGAAG	CGGAAGAGC	8299

WHAT IS CLAIMED IS:

1. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell *ex vivo* comprising the steps of providing a recombinant adeno-associated virus comprising: (a) the DNA of at least a portion of the genome of an adeno-associated virus which portion is capable of transducing a selected gene into a target cell in the absence of cell division; and (b) a selected gene operatively linked to regulatory sequences directing its expression, said gene flanked by the DNA of (a) and capable of expression in the target cell; infecting said target cells with said recombinant adeno-associated virus; contacting said infected cells with an agent which facilitates the conversion of said ss recombinant virus to its double stranded form, wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus into said target cell.
2. The method according to claim 1 wherein said agent is a helper virus comprising a selected gene encoding a polypeptide which can enhance the conversion of said single-stranded recombinant virus to double-stranded recombinant virus or functional fragment thereof, said helper virus capable of transducing said selected gene into the target cell in the absence of cell division.
3. The method according to claim 2 wherein said contacting step comprises co-infecting said target cell with said helper virus.
4. The method according to claim 3 wherein said helper virus is an adenovirus.

5. The method according to claim 2 wherein said selected gene is the adenovirus E4 gene or a functional fragment thereof.

6. The method according to claim 2 wherein said adenovirus contains two selected genes or functional fragments thereof, said genes being the adenovirus E4 gene and E1 gene.

7. The method according to claim 5 or 6 wherein said functional fragment of said E4 gene comprises the open reading frame 6 of E4.

8. The method according to claim 1 wherein said recombinant adeno-associated virus further comprises a second selected gene operatively linked to regulatory sequences capable of directing expression of said second gene, said second gene capable of facilitating the conversion of said single stranded recombinant virus to its double stranded form upon expression, and capable of co-expression with said first selected gene in the target cell, whereby the second gene product is expressed in the target cell, wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus in said target cell.

9. The method according to claim 8 wherein the regulatory sequences directing expression of said second gene comprise an inducible promoter, and wherein expression of said second gene occurs in the presence of an inducing agent.

10. A recombinant adeno-associated virus comprising (a) the DNA of at least a portion of the genome of an adeno-associated virus which portion is capable of transducing at least two selected genes or functional fragments thereof into a target cell in the absence of cell division; (b) a first selected gene operatively linked to regulatory sequences directing its expression, (c) a second selected gene operatively linked to regulatory sequences capable of directing expression of said second gene, said second gene capable of facilitating the conversion of said single stranded recombinant virus to its double stranded form upon expression, said first and said second genes flanked by the DNA of (a), said first and second gene capable of co-expression in the target cell.

11. The recombinant virus according to claim 10 wherein said second gene is selected from the group consisting of an adenovirus E4 gene, ORF6 of E4 and a functional fragment thereof.

12. The recombinant virus according to claim 10 wherein said second gene is the adenovirus E1 gene or a functional fragment thereof.

13. The recombinant virus according to claim 10 wherein the regulatory sequences directing expression of said second gene comprise an inducible promoter, and wherein expression of said second gene occurs in the presence of an inducing agent.

14. The recombinant virus according to claim 10 further comprising (d) an additional selected gene operatively linked to regulatory sequences capable of directing expression of said gene, said additional gene and said second gene capable of jointly facilitating the conversion of said single stranded recombinant virus to its double stranded form upon expression of both said second and additional genes, said first, second and additional genes flanked by the DNA of (a), and capable of co-expression in the target cell.

15. The recombinant virus according to claim 14 wherein said second gene is the adenovirus E4 gene or a functional fragment thereof and said additional gene is the adenovirus E1 gene or a functional fragment thereof.

16. The recombinant virus according to claim 15 wherein said functional fragment of E4 is the ORF6 sequence.

17. The recombinant virus according to claim 15 wherein the regulatory sequences directing expression of said additional gene comprise an inducible promoter, and wherein expression of said additional gene occurs in the presence of an inducing agent.

18. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell comprising the steps of: providing a recombinant adeno-associated virus of claim 10 through 16; infecting said target cells with said recombinant virus; and culturing said infected cells under conditions which enable expression of said selected genes in said target cell,

wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus in said target cell.

19. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell comprising the steps of: providing a recombinant adeno-associated virus of claim 17; infecting said target cells with said recombinant virus; and contacting said target cell with an inducing agent which induces the expression of said second or additional gene in said target cell, wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus in said target cell.

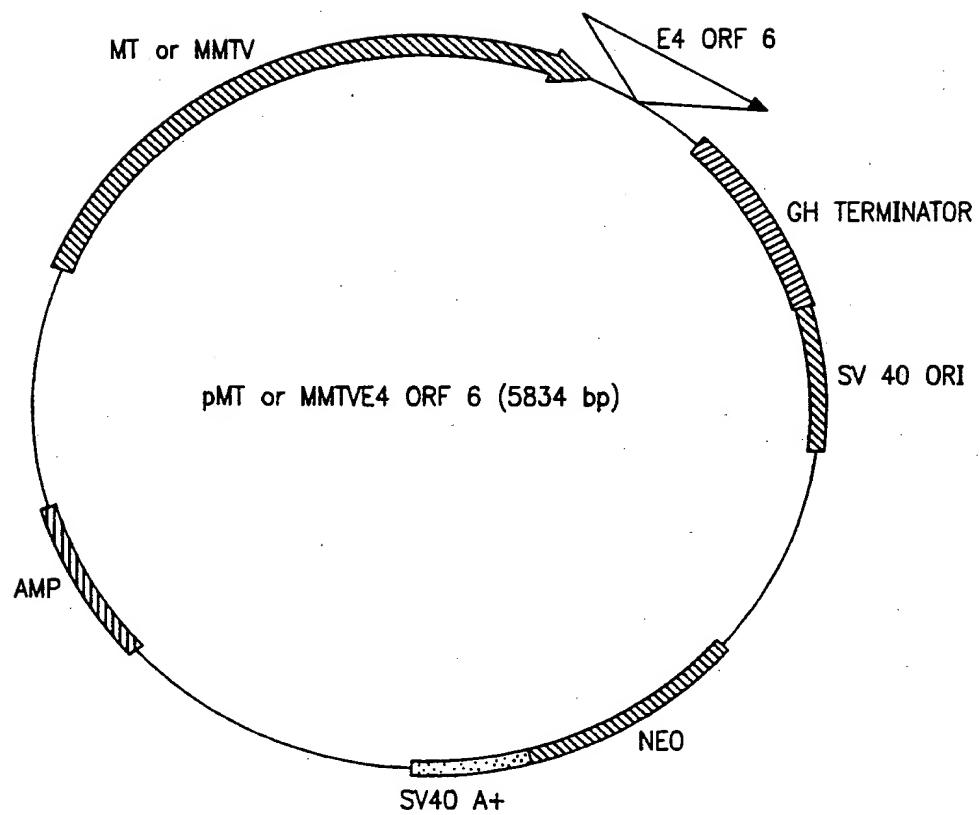
20. A pharmaceutical composition comprising a recombinant adeno-associated virus and an agent which facilitates the conversion of said ss recombinant virus to its double stranded form in a target cell.

21. The composition according to claim 20 wherein said virus is selected from the group consisting of the recombinant viruses of claims 10 - 16.

22. The composition according to claim 20 wherein said virus is the recombinant virus of claim 17 and further comprising an inducing agent which induces the expression of said second or additional gene in said recombinant virus.

23. A mammalian cell transduced with the recombinant adeno-associated viruses of claims 10-17.

1 / 11

**FIG. 1**

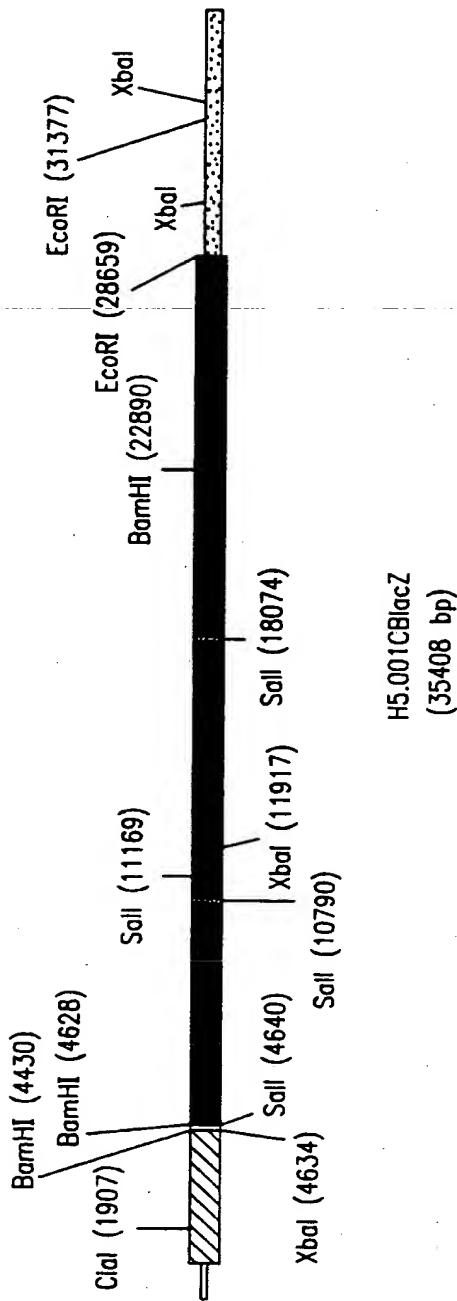
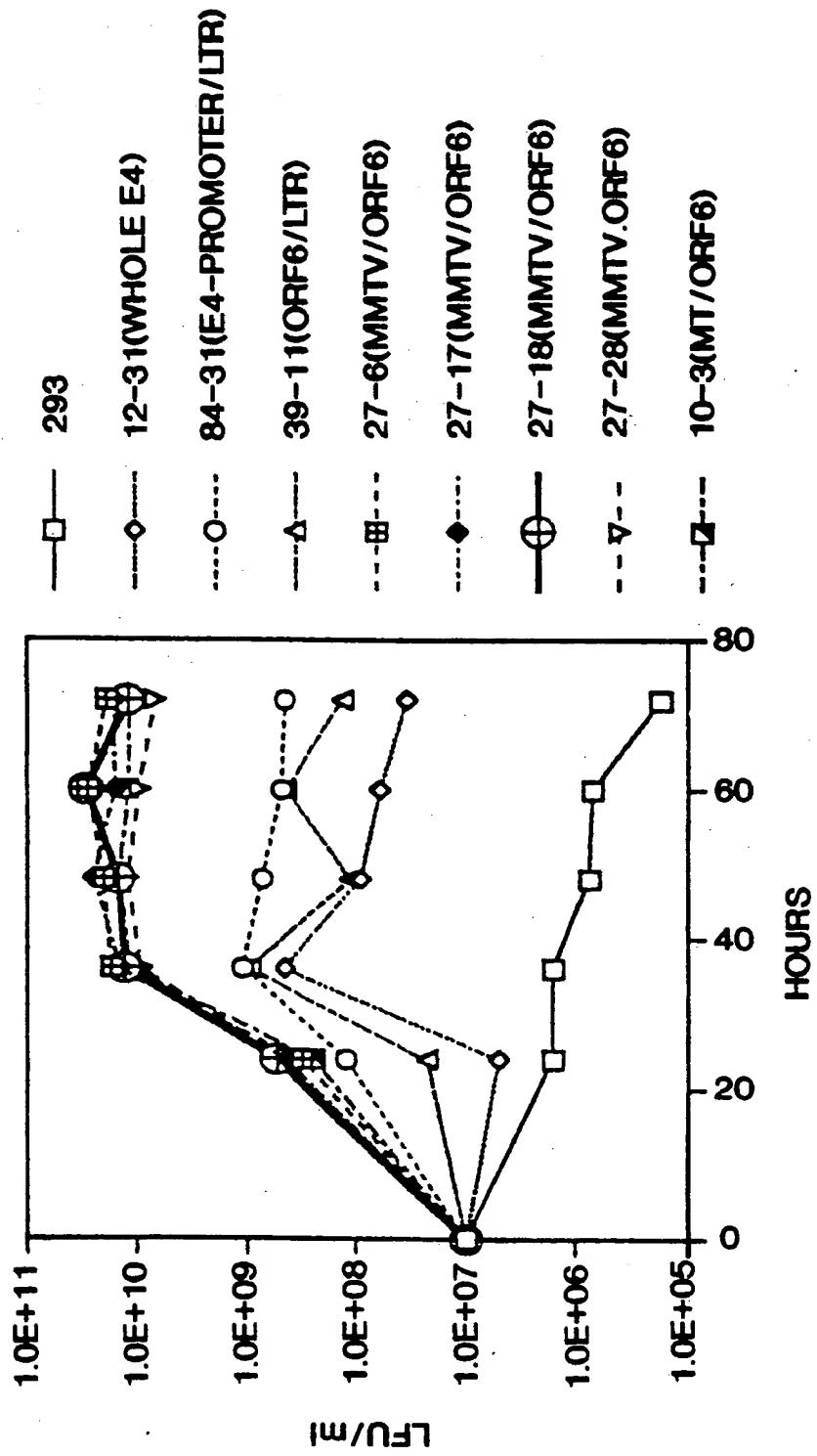


FIG. 2

FIG. 3



4/11

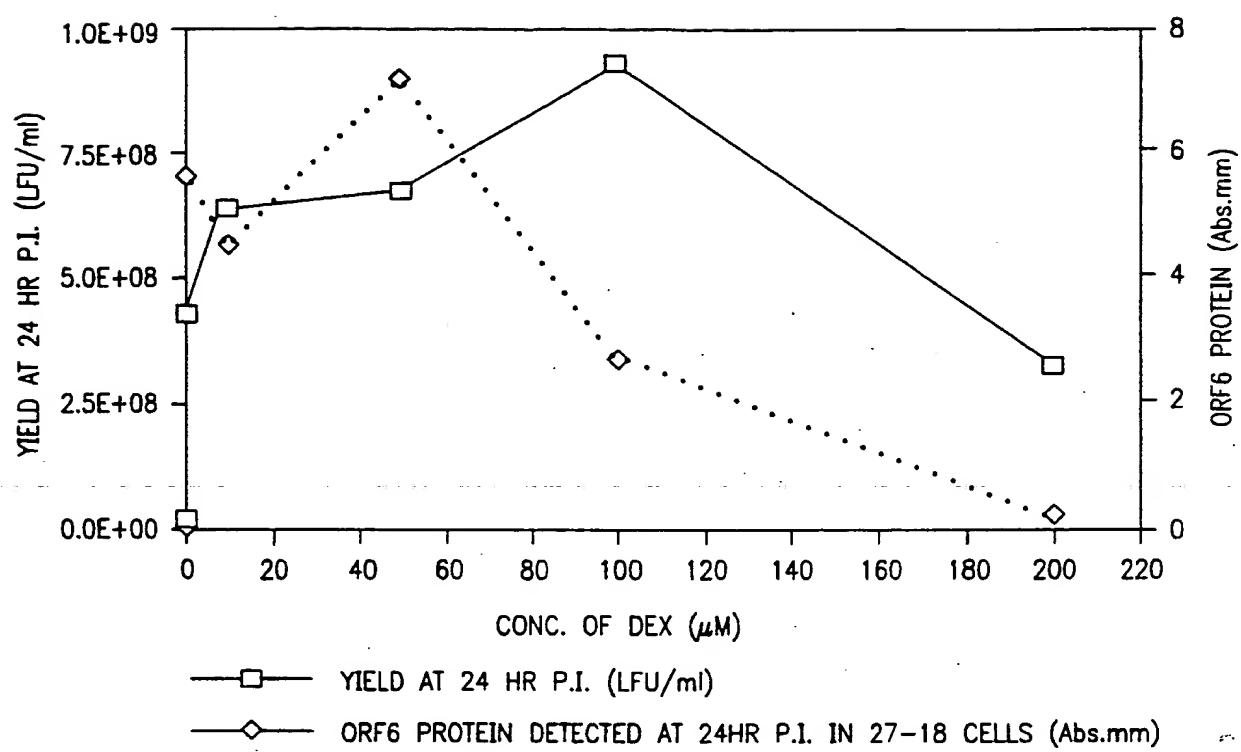


FIG. 4A

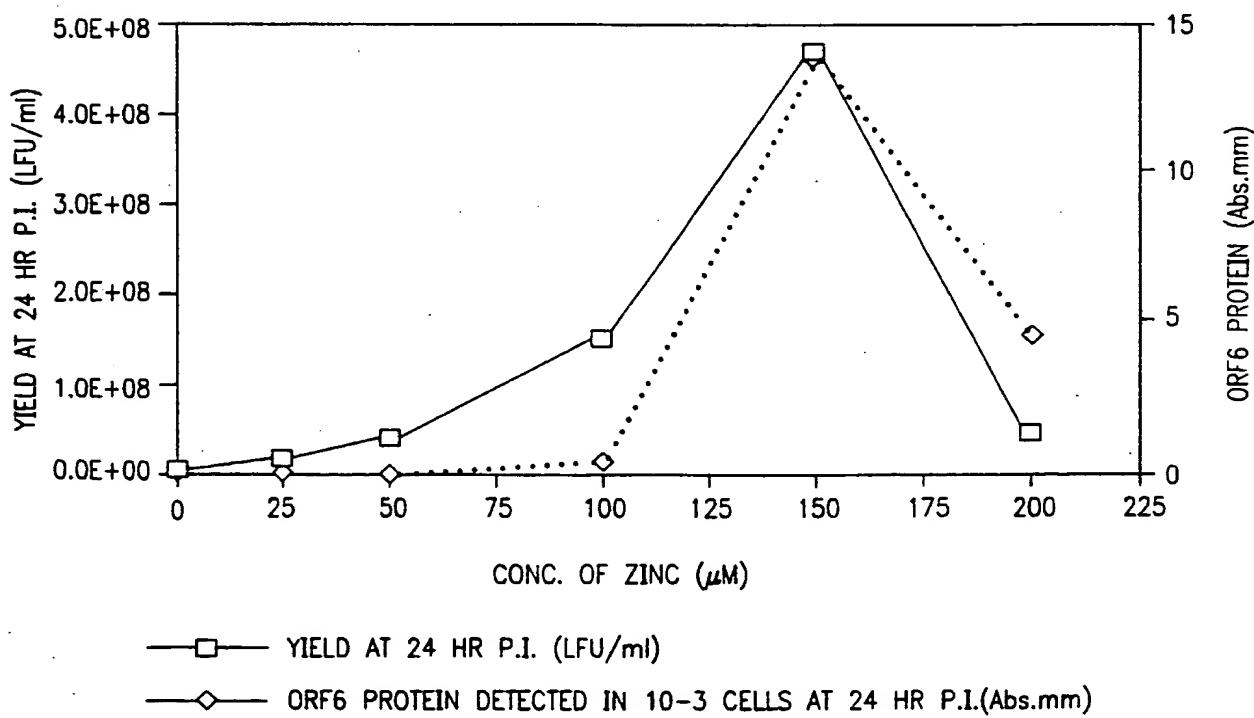


FIG. 4B

5/11

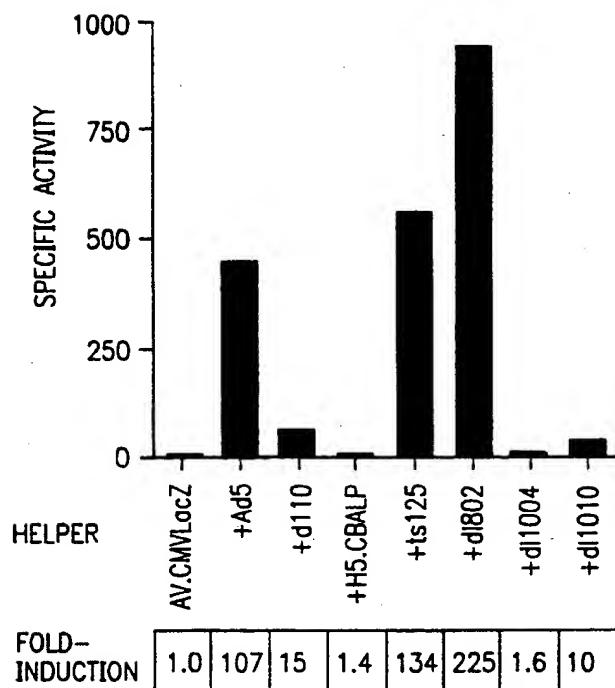


FIG. 5A

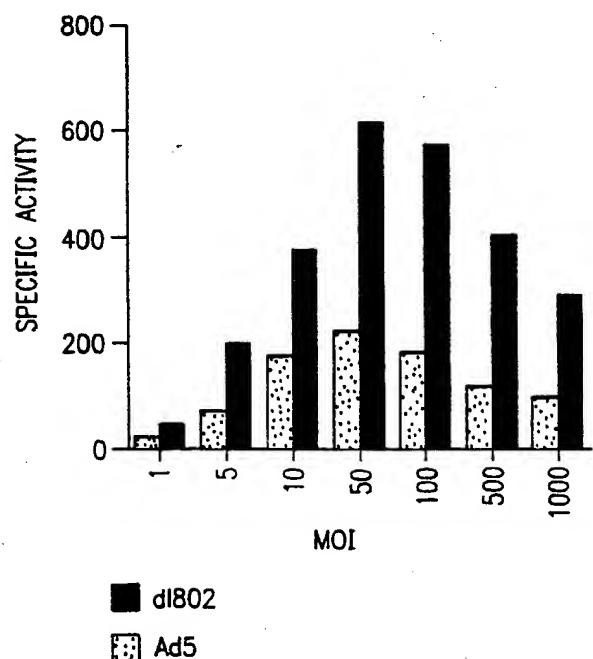
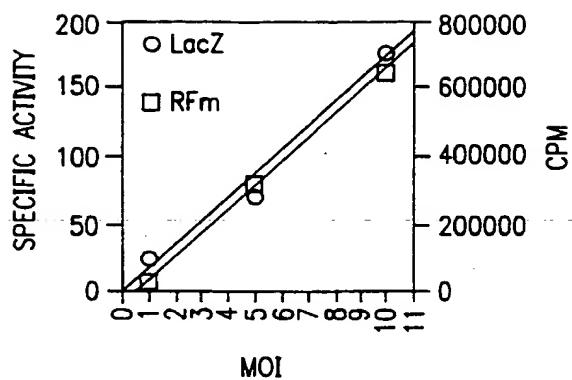
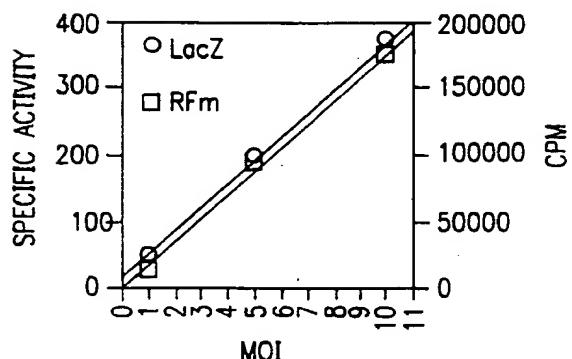


FIG. 5B

6/11

**FIG. 6A****FIG. 6B**

7/11

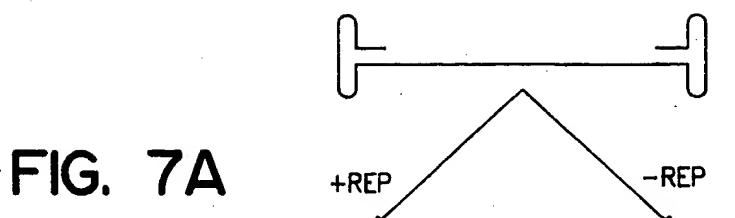
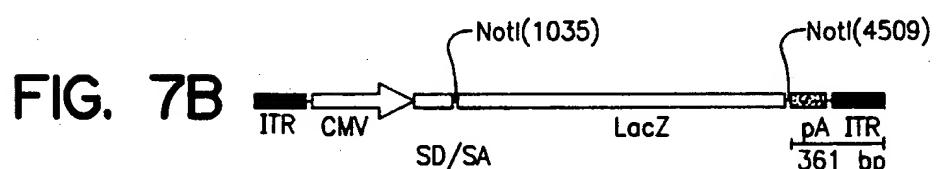
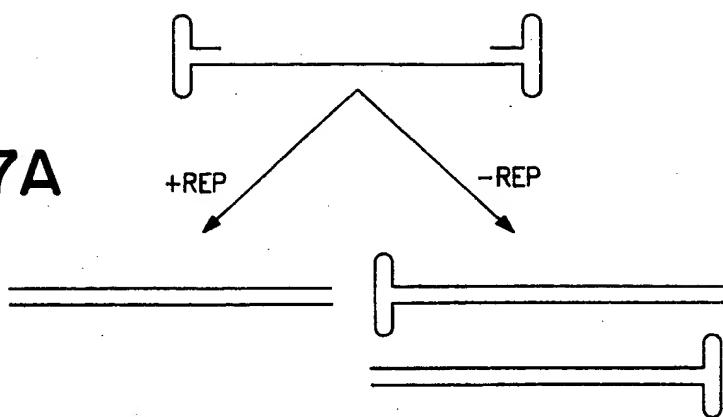
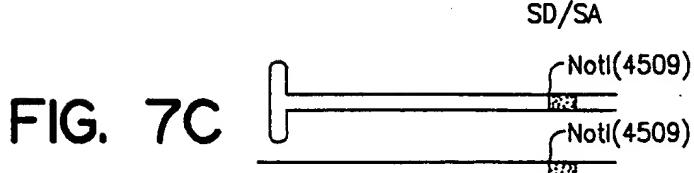
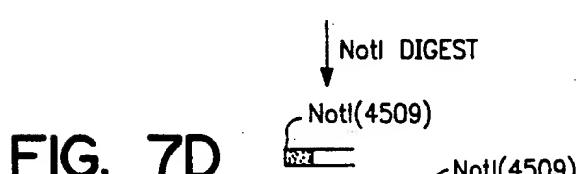
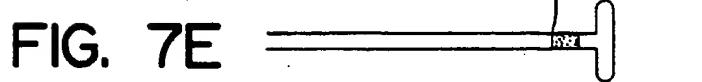
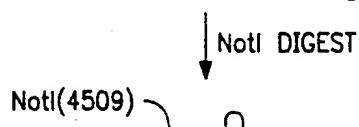
**FIG. 7A****FIG. 7B****FIG. 7C****FIG. 7D****FIG. 7E****FIG. 7F**

FIG. 8A

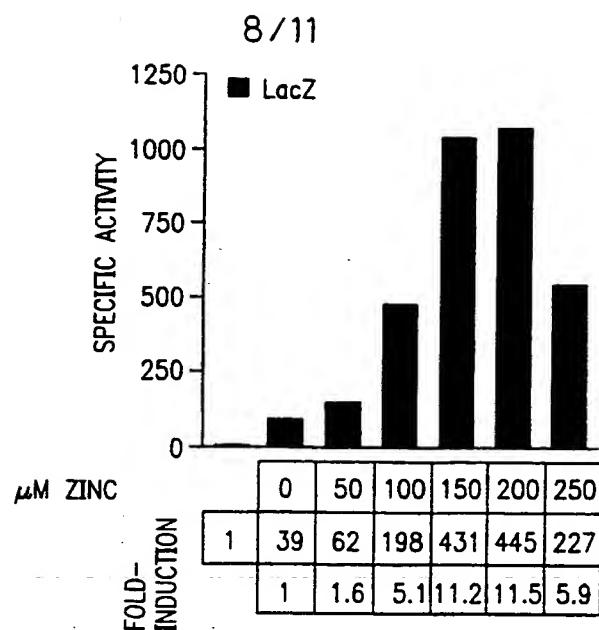


FIG. 8B

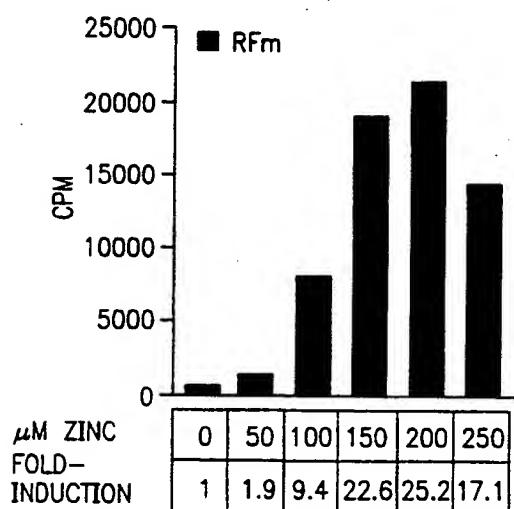
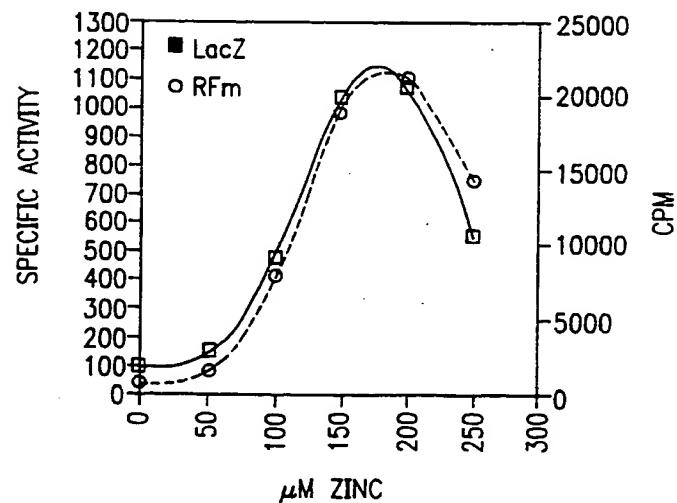


FIG. 8C



9 / 11

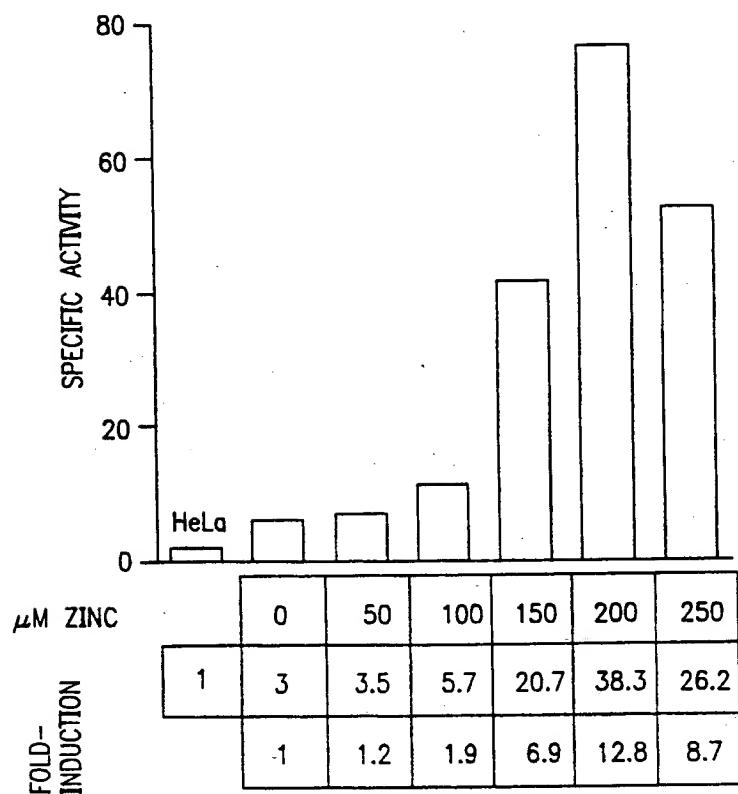


FIG. 9

10/11

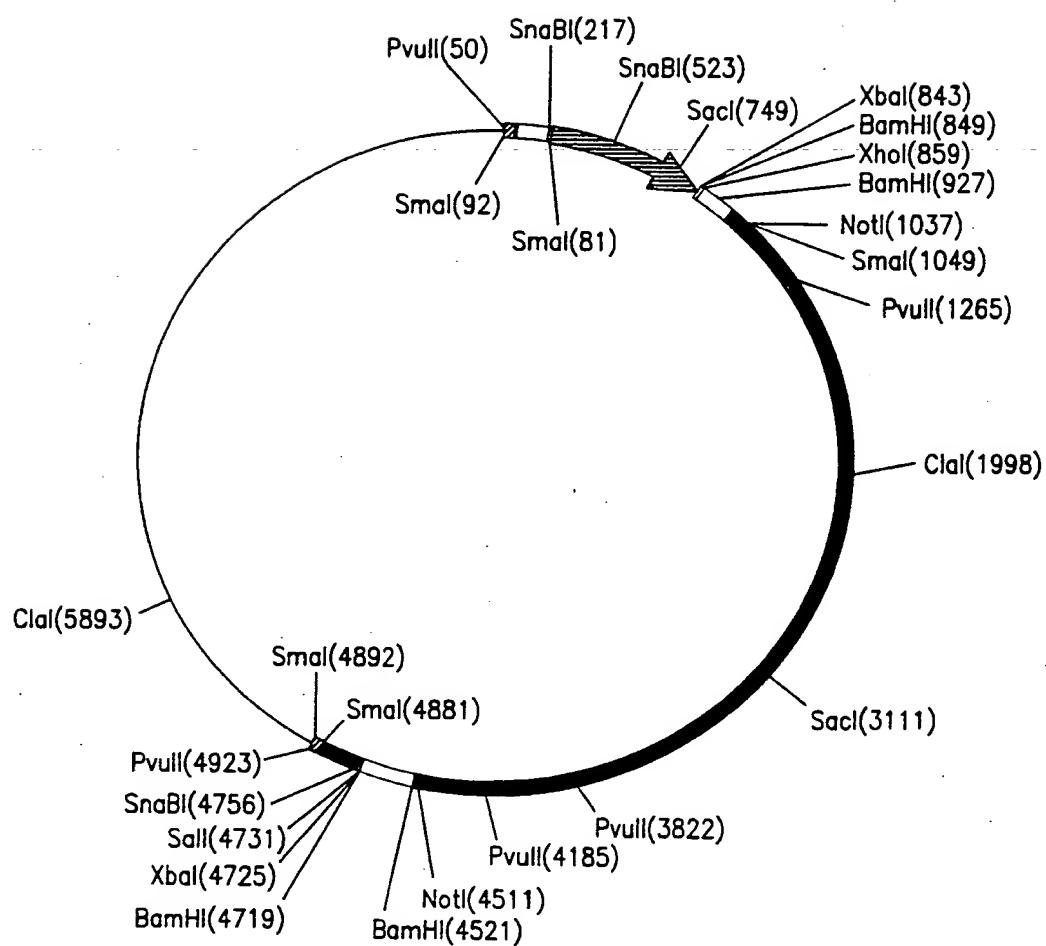


FIG. 10

11 / 11

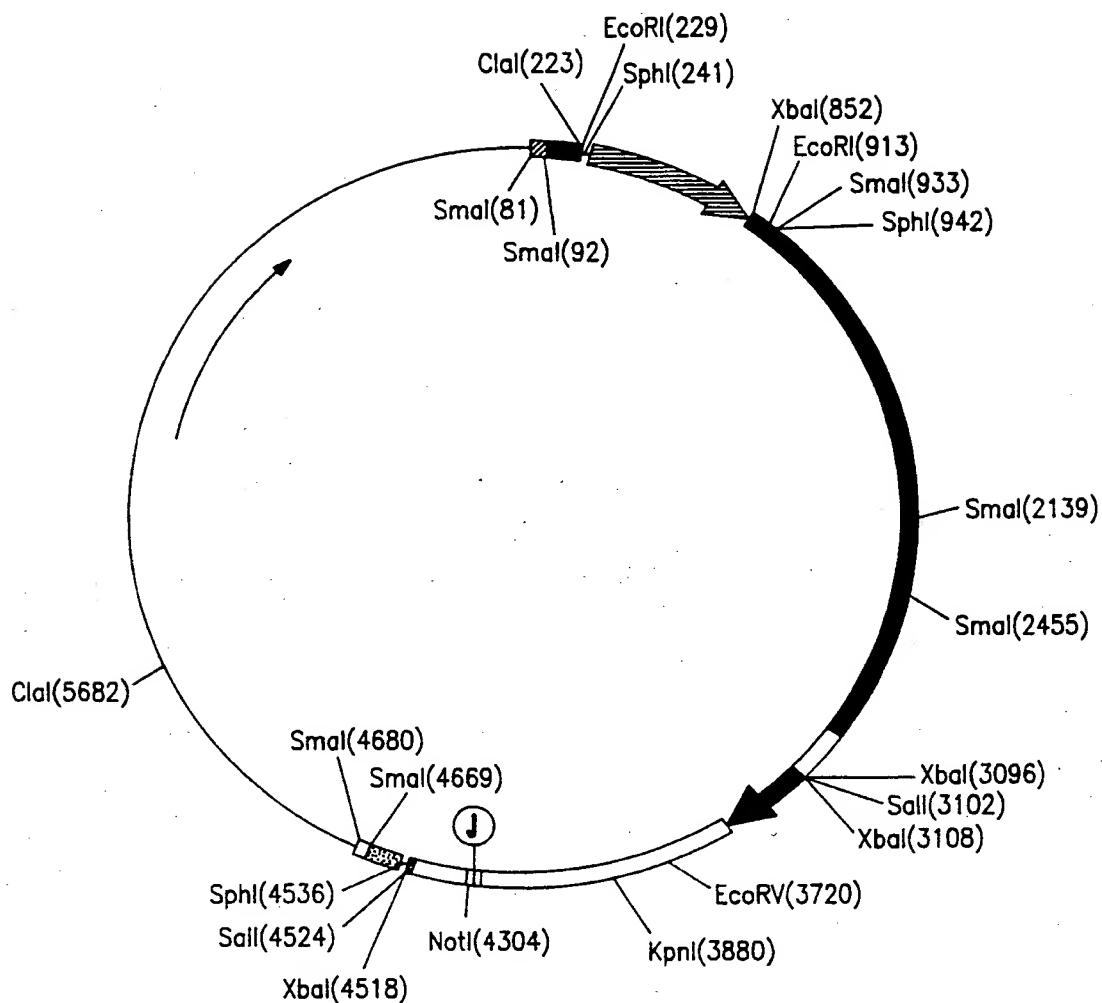


FIG. 11



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US96/10245		Robindale Apartment F5, 1925 Lawrence Road, Havertown, PA 19083 (US).	
(22) International Filing Date: 4 June 1996 (04.06.96)		(74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).	
(30) Priority Data: 08/462,014 5 June 1995 (05.06.95) 08/549,489 27 October 1995 (27.10.95)		US US	(81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(60) Parent Applications or Grants (63) Related by Continuation US 08/462,014 (CIP) Filed on 5 June 1995 (05.06.95) US 08/549,489 (CIP) Filed on 27 October 1995 (27.10.95)		Published With international search report.	
(71) Applicant (for all designated States except US): THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; 133 South 36th Street, Philadelphia, PA 19104-3246 (US).		(88) Date of publication of the international search report: 5 June 1997 (05.06.97)	
(72) Inventors; and (75) Inventors/Applicants (for US only): WILSON, James, M. [US/US]; 1350 N. Avignon Drive, Gladwyne, PA 19035 (US). FISHER, Krishna, J. [US/US]; 4006 Pine Street, Philadelphia, PA 19104 (US). GAO, Guang-Ping [CN/US];			
(54) Title: RECOMBBIANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS, CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF			
(57) Abstract			
<p>An adenovirus E1/E4 expressing packaging cell line is provided, which permits the generation of recombinant adenoviruses deleted in both gene regions. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell is provided by infecting a target cell with a recombinant AAV comprising a selected transgene under the control of regulatory sequences. The infected cell is contacted with an agent which facilitates the conversion of single stranded recombinant virus to its double stranded form.</p>			

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INTERNATIONAL SEARCH REPORT

Inte Application No
PCT/US 96/10245

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6	C12N15/86	C12N15/87	C12N5/10	A61K35/76	A61K48/00
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	J.VIROLOGY, vol. 70, no. 1, January 1996, pages 520-532, XP000570468 FISHER, K.J. ET AL.: "Transduction with recombinant Adeno-Associated Virus for gene therapy is limited by leading-strand synthesis" see the whole document ---	1-7
P,Y		8-23
P,X	J.VIROLOGY, vol. 70, no. 3, March 1996, pages 1845-1854, XP002027273 WEITZMAN, M.D. ET AL.: "Recruitment of wild-type and recombinant Adeno-Associated Virus into Adenovirus replication centers" see the whole document ---	1-7
P,Y		8-23 -/-

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Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1997	25. 03. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl. Fax. (+ 31-70) 340-3016	Authorized officer Donath, C

INTL. NATIONAL SEARCH REPORT

Int'l. Application No.
PCT/US 96/10245

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 95 20671 A (RHONE-POULENC RORER S.A.) 3 August 1995 see page 2, line 33 - page 3, line 30 see page 5, line 28 - page 7, line 24; examples 2-5 ---	1-7
P,X	WO 96 13598 A (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 9 May 1996 see page 4, line 23 - page 5, line 25 see page 8, line 1 - page 15, line 13 see page 18, line 28 - page 19, line 13 ---	1-7
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(54) Title: RECOMBINANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS, CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF			
(57) Abstract An adenovirus E1/E4 expressing packaging cell line is provided, which permits the generation of recombinant adenoviruses deleted in both gene regions. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell is provided by infecting a target cell with a recombinant AAV comprising a selected transgene under the control of regulatory sequences. The infected cell is contacted with an agent which facilitates the conversion of single stranded recombinant virus to its double stranded form.			

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